THE BIOCHEMICAL JOURNAL

THE BIOCHEMICAL JOURNAL

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

A. G. OGSTON (*Chairman*)
E. P. ABRAHAM
H. R. V. ARNSTEIN
S. R. ELSDEN
Q. H. GIBSON
C. H. GRAY
D. C. HARRISON
C. LONG
H. J. ROGERS (*Deputy Chairman*)
L. W. MAPSON
R. MARKHAM
N. H. MARTIN
R. J. PENNINGTON
J. J. SCOTT
I. D. E. STOREY
W. V. THORPE
W. J. WHELAN

Secretary to the Board: F. CLARK

VOLUME 66, 1957

CAMBRIDGE
AT THE UNIVERSITY PRESS
1957

Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)
and published by the Cambridge University Press
Bentley House, 200 Euston Road, London, N.W. 1
American Branch: 32 East 57th Street, New York 22, N.Y.
Agent in Canada: The Macmillan Company of Canada Limited

Science
QP
501
.B57
V.66
1957

THIS P

MAY 19

A

THIS PART CONTAINS REVISED SUGGESTIONS TO AUTHORS

MAY 1957, VOLUME 66, No. 1

UNIVERSITY OF MICHIGAN
JUN X 6 1957
SCIENCE LIBRARY

THE BIOCHEMICAL JOURNAL

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

A. G. OGSTON (*Chairman*) — H. J. ROGERS (*Deputy Chairman*)
E. P. ABRAHAM — R. MARKHAM
H. R. V. ARNSTEIN — N. H. MARTIN
S. R. ELSDEN — D. M. NEEDHAM
Q. H. GIBSON — R. J. PENNINGTON
C. H. GRAY — J. J. SCOTT
D. C. HARRISON — I. D. E. STOREY
C. LONG — W. V. THORPE
L. W. MAPSON — W. J. WHELAN

Secretary to the Board: F. CLARK

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W. 1
AMERICAN BRANCH: 32 EAST 57TH STREET, NEW YORK 22, N.Y.

Price 25s. net (U.S.A. $4.25)

The *Biochemical Journal* is conducted by the **Biochemical Society** and is published by the Cambridge University Press. The *Journal* is issued in three volumes per year. Each volume consists of approximately 700 pages, published in four parts at intervals of one month.

The Biochemical Society was instituted solely for the advancement of the Science of Biochemistry, and holds meetings at different centres for the communication of original papers, and for the discussion of current problems. Persons interested in Biochemistry are elegible for election. Members whose subscriptions are not in arrears are entitled to receive the *Biochemical Journal* without further payment. Further information may be obtained from the Honorary Secretaries, Professor F. L. Warren, Department of Biochemistry, London Hospital Medical College, Turner Street, London, E. 1, and Dr C. E. Dalgliesh, Postgraduate Medical School of London, Ducane Road, Hammersmith, London, W. 12, or from the Honorary Treasurer, F. A. Robinson, Esq., c/o Allen and Hanburys, Ltd., Bethnal Green, London, E. 2.

Subscriptions to the *Biochemical Journal*. For non-members of the Biochemical Society the subscription to the *Biochemical Journal* is £4 per volume, payable in advance to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, or to any bookseller. In the United States of America the *Journal* may be purchased from the Cambridge University Press American Branch, 32 East 57th Street, New York, 22 (subscription price $13.50 per volume).

Communications respecting the sale of single issues or back numbers of the *Journal*, Volumes 20–65, should be addressed to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1. Volumes 1–15 have been reproduced and are available from William Dawson & Sons, Ltd., 4 Duke Street, Manchester Square, London, W. 1, at the prices given below.

Claims for the replacement of *Journals* lost in transmission will not be entertained if they are received later than three months after the date of the posting of the *Journal*.

Prices of back numbers of the *Journal*.

Volumes 1–15 complete.	£82. 10*s*. bound, £75 unbound
Separate volumes.	£6 each bound, £5. 10*s*. each unbound

Reduced terms for Volumes 1–15 are available to members of the Biochemical Society, who should apply to the Honorary Secretaries.

	In paper cover
Volumes 20–28.	£8 per volume of 6 parts at £1. 12*s*. each if sold separately
Volumes 29 & 35.	£8 per volume
Volume 33 Nos. 8–12 only.	16*s*. per number
Volume 33 Index.	2*s*. 6*d*. net
Volume 34 Nos. 1, 3–7 & 12.	16*s*. per number
Volume 34 Nos. 8 & 9, 10 & 11.	£1. 12*s*. per double number
Volume 36.	£5 per volume of 12 parts at 10*s*. each if sold separately
Volume 39 (less Index, out of print).	£5 per volume of 5 parts at £1. 4*s*. each if sold separately
Volume 40.	£5 per volume of 6 parts at £1 each if sold separately
Volumes 41–43.	£5 per volume of 4 parts at £1. 10*s*. each if sold separately
Volumes 44–47, 50–51.	£5 per volume of 5 parts at £1. 4*s*. each if sold separately
Volumes 52–54.	£4 per volume of 4 parts at £1 each if sold separately
Volume 55.	£5 per volume of 5 parts at £1 each if sold separately
Volume 56–64.	£4 per volume of 4 parts at £1 each if sold separately
Volume 65.	£5 per volume of 4 parts at £1 5*s*. each if sold separately
Index to Volumes 11–20.	10*s*. net
Index to Volumes 21–30.	15*s*. net
Index to Volumes 31–40.	£1. 15*s*. net
History of the Biochemical Society.	2*s*. 6*d*. net

CONTENTS OF VOLUME 66, No. 1, 1957

(*All rights reserved*)

PAGE

Suggestions to authors 1

The preparation of [16-^{3}H]progesterone. By W. H. Pearlman 17

The synthesis of serum albumin and tissue proteins in slices of rat liver and liver tumour. By P. N. Campbell and N. E. Stone. (With seven figures) 19

Studies of sebum. 8. Observations on the squalene and cholesterol content and the possible functions of squalene in human sebum. By B. Boughton, R. M. B. MacKenna, V. R. Wheatley and A. Wormall. (With five figures) 32

Metabolism of polycyclic compounds. 11. The conversion of naphthalene into 2-hydroxy-1-naphthyl sulphate in the rabbit. By E. Boyland and P. Sims 38

A nitrogenous derivative of lactose from lactating rat mammary gland. By R. Heyworth and J. S. D. Bacon 41

Addendum—The breakdown of the lactose derivative by bacteria. By M. Shilo 48

The dialysis of brain pyrophosphatase. By J. J. Gordon 50

Potassium uptake of *Alcaligenes faecalis*. By H. A. Krebs, R. Whittam and R. Hems 53

16α-Hydroxyoestrone in the urine of pregnant women. By G. F. Marrian, K. H. Loke, E. J. D. Watson and M. Panattoni. (With one figure) 60

Synthesis of porphyrins by cell suspensions of *Tetrahymena vorax*: effect of members of the vitamin B group. By J. Lascelles. (With two figures) 65

The biochemistry of aromatic amines. 3. Enzymic hydroxylation by rat-liver microsomes. By J. Booth and E. Boyland. (With three figures) 73

The study of steady-state concentrations of internal solutes of mitochondria by rapid centrifugal transfer to a fixation medium. By W. C. Werkheiser and W. Bartley. (With three figures) 79

Steroids and other lipids of pregnant goat's urine. By W. Klyne and A. A. Wright. (With one figure) 92

Protein synthesis in ripening pea seeds. 1. Analysis of whole seeds. By I. D. Raacke. (With five figures) 101

Protein synthesis in ripening pea seeds. 2. Development of embryos and seed coats. By I. D. Raacke. (With two figures) 110

Protein synthesis in ripening pea seeds. 3. Study of the pods. By I. D. Raacke. (With two figures) . 113

The effect of parathormone on the ^{32}P uptake by the bones of rabbits and cats. By J. Dawson, S. M. Weidmann and H. G. Jones 116

The effect of X-radiation *in vivo* on protein and nucleoprotein metabolism in the rat. By J. E. Richmond, M. G. Ord and L. A. Stocken 123

The mechanism of hydrolysis of adenosine di- and tri-phosphate catalysed by potato apyrase. By M. Cohn and G. A. Meek. (With one figure) 128

Inositol in seminal plasma. By E. F. Hartree. (With two figures) 131

Tissue components of the domestic fowl. 1. The D-glucose content of whole blood. By D. J. Bell . 137

The mechanism of the reaction between di-(2-chloroethyl) sulphone (mustard-gas sulphone) and amino acids. By G. E. Francis, D. E. Richards and A. Wormall 142

The quantitative estimation of pyruvic and α-oxyglutaric acids by paper chromatography in blood, urine and cerebrospinal fluid. By B. McArdle 144

Studies on the particulate components of rat mammary gland. 1. A method for determining the composition of the retained fluid. By A. L. Greenbaum and T. F. Slater. (With three figures) 148

PAGE

Studies on the particulate components of rat mammary gland. 2. Changes in the levels of the nucleic acids of the mammary glands of rats during pregnancy, lactation and mammary involution. By A. L. Greenbaum and T. F. Slater 155

Studies on the particulate components of rat mammary gland. 3. The relationship between enzyme activity and particle counts in mammary gland suspensions. By A. L. Greenbaum and T. F. Slater . . 161

Studies on pituitary adrenocorticotropin. 2. Oxidation–reduction properties of the hormone. By M. L. Dedman, T. H. Farmer and C. J. O. R. Morris. (With two figures) 166

The estimation of serum protein-bound iodine by alkaline incineration. By J. D. Acland. (With four figures) 177

Biochemistry of the wood-rotting fungi. 9. Volatile metabolic products of *Stereum subpileatum* Berk. & Curt. By J. H. Birkinshaw, P. Chaplen and W. P. K. Findlay 188

Proceedings of the Biochemical Society

Pitfalls in the interpretation of Ouchterlony gel plates. By J. G. Feinberg 1P

Complexity of β-glucanases from *Aspergillus niger*. By B. A. Stone 1P

The hydroxylation of nicotinic acid by *Pseudomonas fluorescens*. By A. L. Hunt, D. E. Hughes and J. M. Lowenstein 2P

Glutathione and formation of mercapturic acid *in vivo*. By M. M. Barnes and S. P. James . . . 3P

Glutathione and formation of mercapturic acid *in vitro*. By H. G. Bray and T. J. Franklin . . . 3P

The absence of effect of soluble silica on the metabolism of rat-tissue slices. By E. V. Rowsell and R. A. Leonard 3P

A powerful inhibitor for β-*N*-acetylglucosaminidase, and some observations on the specificity of the enzyme. By C. A. Marsh and G. A. Levvy 4P

The galacturonidase activity of α- and β-glucuronidases. By C. A. Marsh and G. A. Levvy . . . 5P

Nucleotides in the skeletal muscle of codling (*Gadus callarias*). By N. R. Jones and J. Murray . . 5P

Incorporation of ^{14}C-amino acids into proteins of the guinea-pig liver microsome fraction *in vitro*. By J. L. Simkin 6P

The protective action of pyridine-2-aldoxime methiodide in organophosphate poisoning. By F. Hobbiger 7P

The sulphydryl groups of myokinase. By O. H. Callaghan 7P

Role of carotenoids in energy migration and photo-oxidation in chlorophyll systems. By F. W. J. Teale and G. Weber 8P

A micro-method for the estimation of tyrosine and its application to tyrosine excretion by phenylketonurics. By J. H. Ottaway 8P

Kinase activity of ultrasonic extracts of *Acetobacter suboxydans*. By J. A. Fewster 9P

Biosynthesis of squalene and cholesterol from DL-β-hydroxy-β-methyl-δ-[2-^{14}C]valerolactone. By J. W. Cornforth, R. H. Cornforth, G. Popják and I. Youhotsky-Gore 10P

β-Hydroxybutyric acid oxidases of rat heart sarcosomes. By R. B. Beechey 10P

A method for preparing cellulose nitrate dialysis tubing. By B. A. Stone 11P

Properties and comparison of some pharmacologically active polypeptides. By A. P. Mathias and M. Schachter 11P

Effect of very high and low protein diets on some aspects of nitrogen metabolism in the obese human and normal rat. By G. L. S. Pawan 11P

Enzymic determination of blood glucose. By A. St G. Huggett and D. A. Nixon 12P

INDEX OF AUTHORS

	PAGE
Acland, J. D.	177
Bacon, J. S. D.	41
Barnes, M. M.	3P
Bartley, W.	79
Beechey, R. B.	10P
Bell, D. J.	137
Birkinshaw, J. H.	188
Booth, J.	73
Boughton, B.	32
Boyland, E.	38, 73
Bray, H. G.	3P
Callaghan, O. H.	7P
Campbell, P. N.	19
Chaplen, P.	188
Cohn, M.	128
Cornforth, J. W.	10P
Cornforth, R. H.	10P
Dawson, J.	116
Dedman, M. L.	166
Farmer, T. H.	166
Feinberg, J. G.	1P
Fewster, J. A.	9P
Findlay, W. P. K.	188
Francis, G. E.	142
Franklin, T. J.	3P
Gordon, J. J.	50
Greenbaum, A. L.	148, 155, 161
Hartree, E. F.	131
Hems, R.	53
Heyworth, R.	41
Hobbiger, F.	7P
Huggett, A. St G.	12P
Hughes, D. E.	2P
Hunt, A. L.	2P
James, S. P.	3P
Jones, H. G.	116
Jones, N. R.	5P
Klyne, W.	92
Krebs, H. A.	53
Lascelles, J.	65
Leonard, R. A.	3P
Levvy, G. A.	4P, 5P
Loke, K. H.	60
Lowenstein, J. M.	2P
McArdle, B.	144
MacKenna, R. M. B.	32
Marrian, G. F.	60
Marsh, C. A.	4P, 5P
Mathias, A. P.	11P
Meek, G. A.	128
Morris, C. J. O. R.	166
Murray, J.	5P
Nixon, D. A.	12P
Ord, M. G.	123
Ottaway, J. H.	8P
Panattoni, M.	60
Pawan, G. L. S.	11P
Pearlman, W. H.	17
Popják, G.	10P
Raacke, I. D.	101, 110, 113
Richards, D. E.	142
Richmond, J. E.	123
Rowsell, E. V.	3P
Schachter, M.	11P
Shilo, M.	48
Simkin, J. L.	6P
Sims, P.	38
Slater, T. F.	148, 155, 161
Stocken, L. A.	123
Stone, B. A.	1P, 11P
Stone, N. E.	19
Teale, F. W. J.	8P
Watson, E. J. D.	60
Weber, G.	8P
Weidmann, S. M.	116
Werkheiser, W. C.	79
Wheatley, V. R.	32
Whittam, R.	53
Wormall, A.	32, 142
Wright, A. A.	92
Youhotsky-Gore, I.	10P

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

The component acids of some Indian fresh-water fish fats. By S. P. Pathak and V. N. Ojha

The analysis of urinary 17-oxo steroids by gradient elution. By A. E. Kellie and A. P. Wade

The isolation of oestrone from the urine of cows in late pregnancy. By G. S. Pope, Mary J. McNaughton and H. E. H. Jones

Sterol metabolism:

1. The biosynthesis of [^{14}C]ergosterol in *Carpenteles brefeldianum* Dodge. By J. Glover and W. M. F. Leat

2. The absorption and metabolism of [^{14}C]ergosterol in the guinea pig. By J. Glover, W. M. F. Leat and R. A. Morton

Incorporation of glycine into glutathione and fibrinogen of rats under adrenaline treatment. By S. B. Henriques, Olga B. Henriques and Fajga R. Mandelbaum

Oxidation of *p*-cresol and related compounds by a *Pseudomonas*. By S. Dagley and M. D. Patel

Studies in the biochemistry of micro-organisms. 101. The colouring matters of species in the *Aspergillus nidulans* group. Part 2. Further observations on the structure of asperthecin. By S. Neelakantan, Anna Pocker and H. Raistrick

The action of di*iso*propyl phosphorofluoridate and other anticholinesterases on amino acids. By R. F. Ashbolt and H. N. Rydon

The catabolism of glucose by strains of *Trypanosoma rhodesiense*. By P. T. Grant and J. D. Fulton

On the determination of the time required for biosynthesis of a single protein molecule: experiments with rat pancreatic ribonuclease. By Valda M. Craddock and C. E. Dalgliesh

The use of the isolated perfused liver to detect alterations to plasma proteins. By A. H. Gordon

Glutamic dehydrogenase in the developing chick embryo. By J. B. Solomon

Interrelationships between lysine and $\alpha\epsilon$-diaminopimelic acid and their derivatives and analogues in mutants of *Escherichia coli*. By Pauline Meadow, D. S. Hoare and Elizabeth Work

The reduction of quinic acid to dihydroshikimic acid by certain lactic acid bacteria. By J. G. Carr, A. Pollard, G. C. Whiting and A. H. Williams

Studies on the carbohydrate metabolism of skin. By C. N. D. Cruickshank, M. D. Trotter and J. R. Cooper

The origin of melezitose: a biochemical relationship between the lime tree (*Tilia* spp.) and an aphis (*Eucallipterus tiliae* L.). By J. S. D. Bacon and Brenda Dickinson

Addendum—X-ray data for melezitose. By O. G. Griffin

The preparation and properties of ribonucleic acids from rat liver. By R. H. Pain and J. A. V. Butler

A study of the cytochromes of *Octopus vulgaris* Lam. By Anna Ghiretti-Magaldi, A. Giuditta and F. Ghiretti

Isolation and properties of malic dehydrogenase from ox-heart mitochondria. By D. D. Davies and E. Kun

Absorption and metabolism of [^{14}C]allethrin by the adult housefly, *Musca domestica* L. By Patricia M. Bridges

β-Glycosidase of potato. By P. Baruah and T. Swain

The metabolism of 8-azapurines. By J. D. Smith and R. E. F. Matthews

Adenine succinic acid and adenylsuccinic acid from mammalian liver: isolation and identification. By W. K. Joklik

The effects of proteolytic enzymes on the hyaluronic acid complex of ox synovial fluid. By B. S. Blumberg and A. G. Ogston

The acid phosphatases of rat liver. By G. A. J. Goodlad and G. T. Mills

The hydrolysis of glucose 1-phosphate by rat-liver extracts. By G. A. J. Goodlad and G. T. Mills

Studies on sulphatases:

16. A soluble preparation of arylsulphatase C of rat-liver microsomes. By K. S. Dodgson, F. A. Rose and B. Spencer

17. The action of surface-active agents on the arylsulphatase C of rat liver. By K. S. Dodgson, F. A. Rose B. Spencer and J. Thomas

THE BIOCHEMICAL JOURNAL

SUGGESTIONS TO AUTHORS CHEMICAL NOMENCLATURE AND ABBREVIATIONS, SYMBOLS, USAGES AND CONVENTIONS

REVISED 1957

SUGGESTIONS TO AUTHORS . . . . . *page* 2
CHEMICAL NOMENCLATURE 6
ABBREVIATIONS, SYMBOLS, USAGES AND CONVENTIONS 8

SUGGESTIONS TO AUTHORS

PAPERS submitted for publication in the *Biochemical Journal* should be written concisely. Special attention is directed to the sections below concerning the preparation of the typescript. Strict observance of these requirements will shorten the interval between the receipt of a paper and its publication. Typescripts which are not concise or which do not conform to the conventions of the *Biochemical Journal* will be returned to the authors for revision.

Papers submitted for publication should be sent to the Secretary to the Editorial Board (*Biochemical Journal*), The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1. Communications about the purchase of reprints should be addressed to the University Press, Cambridge.

Abstracts. Authors should submit with their typescript an abstract suitable for inclusion in *International Abstracts of Biological Sciences*. This abstract will not appear in the *Biochemical Journal* but will be edited before being passed for publication in the *Abstracts*.

The abstract should outline as briefly as possible the results and definitive conclusions of the work submitted. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words. The abstract should be typed in double spacing on a separate quarto sheet in the following form: title; name(s) of author(s); *Biochem. J.* (space for year, volume and page reference); address (for reprint applications); abstract. For example:

> The metabolism of short-chain fatty acids in the sheep. 4. The pathway of propionate metabolism in rumen epithelial tissue. R. J. Pennington and T. M. Sutherland. *Biochem. J.* 1956, **63**, 618–628 (Rowett Research Institute, Bucksburn, Aberdeenshire)—(Abstract follows).

General. It is the policy of the *Biochemical Journal* to publish papers in all fields of biochemistry—plant, animal and microbiological—provided that they describe results which make a new and fundamental contribution to biochemical knowledge, or that they describe methods applicable to biochemical problems. Submission of a paper to the Editorial Board will be held to imply that it reports unpublished work, that it is not under consideration for publication elsewhere, and that if accepted for the *Biochemical Journal* it will not be published elsewhere in the same form, either in English or in any other language, without the consent of the Editorial Board.

Contributors who reside abroad may nominate somebody in Great Britain who is willing to correct the proofs of their papers. Proofs are also sent to all authors residing abroad, if necessary by airmail, whether or not they have nominated a proof reader in Great Britain; if these are returned immediately, it will normally be possible to incorporate corrections in the final proof. The method of correcting proofs given in B.S. 1219 or B.S. 1219C (obtainable from the British Standards Institution) is preferred.

Papers should be headed by an informative title, by the names of the authors and by the name and address of the Laboratory where the work was performed. Female authors should use one given name and the surname, and male authors should use initials and surname only. Descriptive material about the author, e.g. Beit Memorial Fellow, or details of financial support, should appear as a footnote on the first page or, preferably, in the acknowledgements at the end of the paper.

Typescripts should bear the name and address of the person to whom the proof of the paper is to be sent, and should give also a shortened version of the title, not exceeding forty-five letters and spaces in length, suitable for a running title in the published pages of the work.

If a paper that has been returned to an author for revision is not resubmitted within six months, it will be deemed to be a new paper and the date of receipt altered accordingly. A revised paper containing a significant amount of new material will also be redated.

A paper should be written only when a piece of work is rounded off. Preliminary or abortive experiments should not be described.

It would help the editors if the author, when submitting a paper which is part of a series, would enclose reprints of the immediately preceding parts.

Form of papers submitted for publication. Papers should be in double-spaced typing on sheets of uniform size with wide margins. Top copies only should be submitted. The paper should be written in English.

The onus of preparing a paper in a form suitable for sending to press lies in the first place with the author. Authors should consult a current issue of the *Journal* in order to make themselves familiar

1924
739
88414

with the typographical conventions, use of cross-headings, lay-out of tables, citation of references, etc. The need for editorial revision of a badly prepared typescript will lead to delay in publication. Papers on specialized subjects should be presented so that they are intelligible to the ordinary reader of the *Journal*. Sufficient information should be included to permit repetition of the experimental work.

Generally, papers should be divided clearly into sections, as follows: (*a*) introduction, containing the reasons for doing the work; (*b*) Experimental methods: with chemical papers the experimental part may appear towards the end, but otherwise should follow the introduction; (*c*) Results: these should be given concisely; the use of both tables and figures to illustrate the same results will only rarely be permitted; only illustrative protocols should be included; (*d*) Discussion: it is desirable that the presentation of the results should be separated from the discussion of their significance; this section should be strictly limited to discussion, and should not recapitulate results; (*e*) a Summary, about 3 % of the length of the paper: the paragraphs of the Summary should be numbered; (*f*) acknowledgements; (*g*) References. The arrangement suggested for sections (*b*)–(*d*) is not binding on authors; other ways of arrangement are sometimes more suitable.

The *Biochemical Journal* uses as a standard for spelling the *Concise Oxford Dictionary of Current English* (Oxford: Clarendon Press).

References. These should be given in the text thus: Barnett & Robinson (1942), (Culbertson & Thomas, 1933). Where part of the volume is published in one year and part in the next year, the year or years should be as given on the title page of the volume. When a paper to be cited has more than two authors, the names of all the authors should be given when reference is first made (except for more than five authors), e.g. (Osborne, Mendel & Ferry, 1919); subsequent citations should appear thus: (Osborne *et al.* 1919). Where more than one paper by the same authors has appeared in one year the reference should be given as follows: Osborne & Mendel (1914*a*); Osborne & Mendel (1914*b*); or Osborne & Mendel (1914*a*, *b*); (Osborne & Mendel, 1914*a*, 1916; Barnett & Robinson, 1942).

At the end of the paper references should be given in alphabetical order, and should include the authors' initials, but not the title of the paper. Titles of journals should be abbreviated in accordance with the system used in the *World List of Scientific Periodicals* (1952, 3rd ed., London: Butterworths Scientific Publications). Examples of such abbreviations will be found in the current numbers of the *Biochemical Journal*. References to books and monographs should include the town of publication and the name of the publisher, as well as the date of publication and the number of the edition to which reference is made. An example of a reference which is in the form of a citation is as follows: Holman, R. T. & Bergström, S. (1951). In *The Enzymes, Chemistry and Mechanism of Action*, vol. 2, part 1, p. 559. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.

Illustrations. A pamphlet has been issued by the Society giving technical directions for the drawing of illustrations. A copy will be found in the *Biochemical Journal* for January 1956. Reprints may be obtained on application to the Editorial Office, price 1*s*. Diagrams which do not conform with these directions may have to be redrawn by the Press and the expense charged to the author. Illustrations should be on separate sheets and packed flat; they should bear the author's name and the title of the paper on the back. Diagrams should be in indian ink and should preferably be drawn on Bristol board, faintly blue-lined graph paper or tracing cloth. Legends and captions should be typed on sheets of paper separate from the illustrations and numbered correspondingly. They should be written so that the general meaning of each illustration can be understood without reference to the text, and so that the exact experimental conditions used to obtain the results illustrated are made clear; reference to the text for the description of experimental details is permissible provided that there is no ambiguity.

When figures show absorption spectra, the legend should give the wavelengths of important maxima.

Illustrations requiring reproduction as half-tone plates should be avoided whenever possible. Their acceptance is considered where their publication is important to the understanding of the text, but the author may be asked to bear the cost.

Chromatograms. Photographs or drawings of paper chromatograms, particularly one-dimensional, are not generally published unless they show the behaviour of new or unidentified substances or the performance of a new solvent system. In paper chromatography, the rate of movement of a substance relative to that of the solvent front is expressed as R_F. In column chromatography R represents (movement of band)/(movement of liquid above surface of column).

Tables. Tables should have headings which make their general meaning comprehensible without reference to the text. Conditions specific to the particular experiment should be stated. Reference to the text for general experimental methods is permissible provided that there is no ambiguity. The units in which the results are expressed,

e.g. g./100 ml., should be given at the top of each column, and not repeated on each line of the table.

Tables should be typed on separate sheets and their approximate position in the text indicated. Words or numerals should be repeated on successive lines: 'ditto' or '„' are not to be used.

Statement of powers in tables and figures. Care is needed where powers are used in table headings and in figures in order to avoid numbers with an inconvenient number of digits. The rule to be followed is that the quantity expressed is to be preceded by the power of 10 by which its value has been multiplied. The units in which the quantity is expressed may not be multiplied by a power of 10; the unit may be changed by the use either of prefixes, such as m or μ, or of another accepted scale such as Å. For example: (i) An entry '2' under heading 10^3k means that the value of k is 0·002; an entry '2' under heading $10^{-3}k$ means that the value of k is 2000. (ii) A concentration 0·00015 M may be expressed as 0·15 under heading 'concn. (mM)' *or* as 150 under heading 'concn. (μM)' *or* as 15 under heading '10^5 concn. (M)', but *not* as 15 under heading 'concn. (M × 10^{-5})'. (iii) Complex quantities are similarly treated; a value for 1/[S] of 200 M^{-1} would appear as '2' under the heading

$$10^{-2}(1/[S])\ (M^{-1}).$$

Statistical treatment of results. It is often unnecessary to publish the individual results of a number of similar experiments. When the object is to determine the value of a quantity or the statistical characteristics of a population, sufficient information is usually conveyed by the following: (i) the *number* of individual experiments; (ii) the *mean value*; (iii) the *standard deviation* (S.D.), *coefficient of variation*, *standard error* (S.E.) or the *standard error of the estimate of mean value* (S.E.M.), as may be appropriate. A convenient form for inclusion in a table is, for example, $263 \pm 2{\cdot}5$ (10), where the numbers represent mean ± S.E. (no. of results).

Where a significant difference is claimed between the means (or other statistics) of two groups of results, an appropriate test of significance should be used.

In representing statistical quantities by symbols, the convention of using Greek symbols (Σ, σ, μ, etc.) for the hypothetical characteristics of the population, and roman symbols (S, s, m, etc.) for actual estimates of their values based on limited samples should be observed.

For the definitions of statistical quantities reference should be made to Kendall, M. G. & Buckland, W. R. (1957). *Dictionary of Statistical Terms*. Edinburgh: Oliver and Boyd Ltd.

Footnotes. These should be avoided in the text as far as possible. Where they must be used, as in tables, reference is made by the symbols * † ‡ § ‖ ¶, in that order.

Moles. The word 'mole' is used with the meaning of 'gram-molecule'. It must not be abbreviated to 'mol.' or 'M'. The submultiples will be printed as 'm-mole' and 'μmole'.

The number of molecular proportions of reagents employed in a reaction, often referred to in everyday language as 'molecules', may be abbreviated in Experimental sections to 'mol.prop.'.

Description of solutions. Solutions of common acids and bases should always be expressed in terms of normality (N), and salts preferably in terms of molarity (M), e.g. N-HCl, 0·1 M-NaH_2PO_4, 0·1 N-acetic acid. Fractional concentrations should preferably be expressed in the decimal system, e.g. 0·25 N-HCl (not N/4 HCl). The term '%' must be used in its correct sense, i.e. g./100 g. of solution. For 'per cent by volume', i.e. ml./100 ml., the term '% (v/v)' may be employed. To indicate that a given weight of substance is contained in 100 ml. of solution, the term '% (w/v)' (weight per volume) may be used. For aqueous solutions of concentration less than 1 %, w/v need not be inserted if it is clear that the concentration is stated in terms of weight of solute.

Metabolic quotients. The symbol Q_x may be used to represent metabolic quotients only in the units μl. of gas X/mg. dry wt. of biological material/hr.; metabolic quotients in any other units, e.g. μmoles of X/mg. dry wt./hr.; μl. of X/mg. of N/sec., may be represented by the symbol q_x; the units for Q or q must be clearly defined at the first mention in each paper. Production and removal of metabolites are indicated by quotients with + and − signs respectively; the signs may be omitted if no confusion can thus arise. Aerobic or anaerobic conditions may be indicated by the superscripts O_2 and N_2 respectively, thus: $Q_{CO_2}^{O_2}$, $Q_{CO_2}^{N_2}$, $q_{CO_2}^{O_2}$, $q_{CO_2}^{N_2}$. When measurements are made in another gas, this may be indicated by the appropriate superscript, e.g. $Q_{CO_2}^{H_2}$; the substrate may, if necessary, be indicated in parentheses following the Q symbol, thus $Q_{CO_2}^{N_2}$ (pyruvate); it is sometimes preferable not to use the special symbols Q_x or q_x, but to state units (e.g. μmoles/g. wet wt./hr.) each time a metabolic quotient is mentioned.

Spectrophotometric results. Spectrophotometric terms and symbols are those proposed by the Society for Analytical Chemistry (see *Analyst*, **1942**, **67**, 164).

Nomenclature of micro-organisms. In the title, at the first mention in the text, and in the Summary of the paper, micro-organisms should be given their full binominal Latin name and underlined. If the organism has been obtained from a recognized collection of micro-organisms, a collection number must be given; alternatively, a strain number or name should be quoted; this should not be underlined. Names of ranks higher than genus (e.g. Eubacteriales, Lactobacilleae) should also not be underlined; nor should generic names used adjectivally (e.g. 'staphylococcal'), nor names of micro-organisms used colloquially (as in, for example, 'all the strains were clostridia', 'most lactobacilli behave thus'). The first (i.e. generic) name should be spelt with a capital letter. Elsewhere in the text, single-letter abbreviations may be given for the generic name provided that ambiguity is not introduced into the paper (e.g. when two genera with the same initial letter are studied; in such papers commonly accepted abbreviations, e.g. *Strep.* and *Staph.*, or, for less well-known organisms, reasonable abbreviations of the author's choice, should be employed). Official Opinions about the conservation or rejection of certain names have been issued and are obtainable from the Editorial Office. If the author selects for stated reasons a name that does not conform to that chosen in the most recent edition of one of the reference books quoted below, the name given in the reference book should be added in brackets after the first mention of the organism in the text, and again in the Summary. Characteristics of the organism that are known to differ from those quoted in the reference book should also be given, since they are essential for subsequent interpretation of the work.

Reference books. Bacteria: Bergey's *Manual of Determinative Bacteriology*. London: Baillière, Tindall and Cox. *Microfungi*: *A Dictionary of the Fungi* by Ainsworth, G. C. & Bisby, G. R. Kew: Imperial Mycological Institute. *Yeasts*: *The Yeasts: a Taxonomic Study* by Lodder, J. & Kreger van Rij, N. J. W. Amsterdam: North Holland Publishing Co.

Collections of micro-organisms. Authors are urged to offer organisms with uncommon characteristics or which are valuable for specific purposes to collections of micro-organisms so that they may be readily available to other workers. The addresses of the British collections of micro-organisms are as follows. *Bacteria of interest in industry or research and those used in microbiological assay*: The National Collection of Industrial Bacteria, Department of Scientific and Industrial Research, Chemical Research Laboratory, Teddington, Middlesex. *Bacteria pathogenic to man or animals*: National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9. *Bacteria related to dairy products*: National Collection of Dairy Organisms, National Institute for Research in Dairying, Shinfield, nr. Reading, Berks. *Fungi*: The Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey. *Yeasts*: The National Collection of Yeast Cultures, The Brewing Industry Research Foundation, Nutfield, Surrey. *Fungi and yeasts pathogenic to man or animals*: The Sub-Department of Medical Mycology, London School of Hygiene and Tropical Medicine, Keppel Street, Gower Street, London, W.C. 1.

Nomenclature of enzymes. Recommendations for the nomenclature of enzymes concerned with fatty acid metabolism are given in *Biochem. J.* (1956), **64**, 782.

Trade names. The names of the manufacturers or suppliers of special apparatus or materials should be given, and also their addresses where these are outside Great Britain or not well known.

CHEMICAL NOMENCLATURE

General. The system of the Chemical Society is usually followed. The fundamental principles of organic chemical nomenclature are given by the Definitive Report of the Organic Commission of I.U.P.A.C. (see *J. chem. Soc.* 1952, p. 5064). The most comprehensive account is given by A. D. Mitchell in *British Chemical Nomenclature* (1948), London: Edward Arnold (Publishers) Ltd., but this is supplemented and in part superseded by the Editorial Reports on Nomenclature in *J. chem. Soc.* 1950, p. 3699; 1951, p. 3516; 1952, p. 5057; 1953, p. 4201; 1954, p. 4714; 1955, p. 4497; 1956, p. 4990.

Detailed proposals for the nomenclature of certain classes of organic compounds are as follows:

Amino acids. See *Biochem. J.* 1948, **42**, 1; 1952, **52**, 1; *J. chem. Soc.* 1951, p. 3522; 1952, p. 5089. The list of Abbreviations, Symbols, Usages and Conventions contains a set of abbreviations marked * for the names of amino acids and their residues taken from a review by Brand, E. & Edsall, J. T. (1947, *Annu. Rev. Biochem.* **16**, 224). These are to be used only to represent the structures of polypeptides (e.g. Gly.Ala = glycylalanine), and the formulae of proteins (cf. the example given by Brand & Edsall) and, in tables and figures, for amino acids. Any abbreviation must be defined at its first mention in each paper.

Carbohydrates. Definitive rules governing the nomenclature of carbohydrates have been agreed between British and U.S. chemists (see *J. chem. Soc.* 1952, p. 5108).

Where oligosaccharides are concerned these definitive names are often too cumbersome for repeated mention in the text of a paper. The coining of trivial names for newly discovered oligosaccharides is considered to be an unsatisfactory remedy for this defect, and to meet the difficulty the Editorial Board has sanctioned the use of shortened names for oligosaccharides which, within the conventions of the system employed, are unambiguous. Examples of the use of such names are to be found in papers by Allen, P. J. & Bacon, J. S. D. (1956). *Biochem. J.* **63**, 200; Hestrin, S., Feingold, D. S. & Avigad, G. (1956). *Biochem. J.* **64**, 340; and Feingold, D. S., Avigad, G. & Hestrin, S. (1956). *Biochem. J.* **64**, 351. These abbreviated names are not to be considered as superseding the internationally agreed names. At its first mention the shortened name of the oligosaccharide should be given in parentheses after the definitive name based on the Rules of Carbohydrate Nomenclature (*J. chem. Soc.* 1952, p. 5108). The conventions followed in assigning the shortened name will be made available to interested authors.

The list of Abbreviations and Symbols contains a set of abbreviations marked † for the names of monosaccharide residues (cf. *J. chem. Soc.* 1952, p. 5121). These are to be used only for the diagrammatic representation of the structures of oligo- and poly-saccharides, and any abbreviation must be defined at its first mention in each paper.

Organophosphorus compounds. See *J. chem. Soc.* 1952, p. 5122.

Organosilicon compounds. See *J. chem. Soc.* 1952, p. 5077.

Steroids. The Ciba Foundation rules, *J. chem. Soc.* 1951, p. 3526, will be used.

Vitamins. See *Biochem. J.* 1952, **52**, 1; *J. chem. Soc.* 1953, p. 4719. Terms such as 'vitamin A' and 'vitamin B_6' may still be used to emphasize the biological activity and not the chemical nature of the material.

Order of prefixes. An alphabetical order will be followed (cf. *J. chem. Soc.* 1950, p. 3699). Syllables indicating multiple substituents, e.g. di-, tri-, do not count in deciding the order.

Use of hyphens and italics. All names are run together except for those of acids, acetals, esters, ethers, glycosides, ketones and salts, which are printed as separate words; hyphens are used to separate numbers, Greek letters or some configurational and italic prefixes from words: e.g. *m*-dinitrobenzene, *ββ*-dimethyl-D-cysteine, 2-*p*-*iso*-propylphenylheptane, ethyl methyl ketone.

Italics are used for certain prefixes so that these shall not be counted as part of the name for indexing, e.g. *iso*, *sec.*-, *tert.*-, *cis*-, *trans*-, *allo*, *cyclo*, *meso*, *o*-, *m*-, *p*-, *dextro*, *laevo*. They are also used for *O*-, *N*-, etc. to indicate an element carrying a substituent, e.g. N^4-acetylsulphanilamide. The following forms are also used: C_{20} acid, an acid containing 20 carbon atoms; C-3 or $C_{(3)}$, the carbon atom numbered 3; *C*-Me, carbon-methyl group.

Use of the abbreviations *o*-, *m*- and *p*- should be confined to cases when they are part of a chemical name, as in *o*-chlorotoluene, toluene-*p*-sulphonic acid, *m*-nitro group. In all other cases *ortho*, *meta*, and *para* should be written in full, as in *ortho* effect, *ortho* substituent, *para* group, *meta*-directing.

Formulae. Elements, groups, inorganic compounds (except water) and chloroform, carbon tetrachloride, carbon disulphide, carbon monoxide

and carbon dioxide may be denoted by symbols in the Experimental portion of the paper and in tables and figures, but not generally in other portions. Diatomic molecules are denoted appropriately, e.g. H_2, O_2, N_2, I_2. For salts it must be stated whether or not the anhydrous material is used, e.g. anhydrous $CuSO_4$, or which of the different crystalline forms is intended, e.g. $CuSO_4,H_2O$; $CuSO_4,5H_2O$.

Formulae should, if possible, be written in one line, since this saves space and expense in printing. Dots are used to represent the links in the main chain; side chains are in parentheses, and condensed main chains are in square brackets, e.g.:

$$CH_2{:}CH\cdot CH(OH)\cdot CH_3;$$

$$H_2N\cdot[CH_2]_3\cdot CH(NH_2)\cdot CO_2H.$$

Structural formulae are normally reproduced from blocks. A careful drawing which can be copied by a draughtsman should be made on a separate sheet. Hetero atoms should be shown in the ring, and aromatic rings must show double bonds.

Optically active isomers. Small capital letters, D-, L- and DL-, are used for optically active compounds and their racemates when the compounds can be correlated sterically with glyceraldehyde or serine. In all other cases (+)-, (−)-, (±)-, or *dextro*, *laevo* and 'optically inactive', are used.

Isotopically labelled organic compounds. The following proposals were made jointly by the Editorial Board of the Biochemical Society and the Editors of the Chemical Society (see *J. chem. Soc.* 1953, p. 4203). Further details, including an extension of the scheme to cover quantitative aspects, may be obtained from the *Biochemical Journal* Editorial Office.

'The symbol for the isotope introduced is placed in square brackets directly attached to the front of the name, as in [^{14}C]urea.

When more than one position in a substance is labelled by means of the same isotope the number of labelled atoms is added as a right-hand subscript (cf. ordinary formulae), as in [$^{14}C_2$]glycollic acid.

When isotopes of more than one element are introduced, their symbols are arranged in alphabetical order, including ^{2}H and ^{3}H for deuterium and tritium respectively.

The isotopic prefix precedes that part of the name to which it refers, as in 2-acetamido-7[^{131}I]-iodofluorene, α-naphth[^{3}H]oic acid ($C_{10}H_7\cdot CO_2{}^3H$), sodium [^{14}C]formate, 1-amino[^{14}C]methyl*cyclo*pentanol ($H_2N\cdot{}^{14}CH_2\cdot C_5H_8\cdot OH$).

When not sufficiently distinguished by the foregoing means, the positions of isotopic labelling are indicated by Arabic numerals, Greek letters, or prefixes (as appropriate), placed within the square brackets and before the symbol of the element concerned, to which they are attached by a hyphen; examples are [1-^{2}H$_1$]ethanol ($H_3C\cdot CH^2H\cdot OH$), [1-^{14}C]aniline, [α-^{14}C]leucine, [*carboxy*-^{14}C]leucine, [*Me*-^{14}C]isoleucine, [6:7-$^{14}C_2$]xanthopterin, [αβ-$^{14}C_2$]maleic anhydride, [1-^{14}C:2-^{13}C]acetaldehyde, [βγ-$^{13}C_2$:^{34}S]methionine, [β-^{14}C:αβ-$^2H_2$:^{15}N]serine.'

Analyses and physical properties. The standard forms for reporting these are shown in the following examples:

The *new compound* (name in italics) had m.p. 175° (decomp.); $[\alpha]_D^{22} + 17 \pm 2°$ in water (*c*, 1·6) (Found: C, 40·8; H, 6·9; N, 11·5; OMe, 26·0. $C_8H_{16}O_6N_2$ requires C, 40·7; H, 6·8; N, 11·9; OMe, 26·3%). The known compound (name in roman) had m.p. 178–179°, unchanged by admixture with an authentic sample kindly supplied by Dr Z (Found: C, 48·6; H, 6·1; OMe, 50·1. Calc. for $C_{10}H_{16}O_7$: C, 48·4; H, 6·4; OMe, 50·0%). *Alternatively*: The known compound had m.p. 178–179°. The mixed m.p. with an authentic sample (m.p. 179–181°) prepared according to X & Y (1932) was 178–180° (Found: C, 49·4; H, 3·8; N, 3·9; loss at 100°, 5·1. Calc. for $C_{28}H_{22}N_2I_2,2H_2O$: C, 49·7; H, 3·9; N, 4·2; H_2O, 5·3%).

Distillation of the product gave a middle fraction (0·3 g.), b.p. 120°/15 mm., n_D^{16} 1·4767; light-absorption max., 226 and 265 mμ (ε 2200 and 2500 respectively) (Found: C, 65·0; H, 8·5. Calc. for $C_{10}H_{16}O_3$: C, 65·2; H, 8·8%). This product was probably a mixture of *A* and *B*.

Melting points. It is desirable to state whether m.p.'s are corrected or uncorrected for the emergent stem of the thermometer. This can often be done conveniently at the beginning of the Experimental section.

Specific optical rotations. It is desirable to give an estimate of the error.

Elementary analyses. Percentages should generally be given to one place of decimals only, since the second place is rarely significant. Elements are given in the order C, H, O, N, Cl, Br, I, F, S, P, and then the remainder in alphabetical order of symbols.

ABBREVIATIONS, SYMBOLS, USAGES AND CONVENTIONS

The following alphabetical list gives abbreviations which may be used in the text of papers, symbols which are primarily for use in mathematical formulae but which may be used in the text, and usages, conventions and spellings adopted.

Abbreviations. New abbreviations should be coined only for unwieldy names, and should not be used at all unless those names occur frequently. All abbreviations must be defined at first mention; alternatively, if many abbreviations are used, they may be grouped together and defined in a separate paragraph at the beginning of the Experimental section.

In the introductory section of the paper and in the Summary abbreviations should be avoided; in the Results and Discussion sections they should be used sparingly. In all these sections abbreviations for units may be used, but not contractions such as 'soln.' or 'recryst.' or chemical symbols as abbreviations for elements, groups or compounds, unless this use is justified by special circumstances.

The abbreviation for the plural of a unit is the same as that for the singular, unless confusion is likely to arise. Thus 'centimetres' is 'cm.' *not* 'cms.'.

Oxidized and reduced coenzymes. The abbreviations GSH and GSSG are used for reduced and oxidized glutathione. DPN and TPN may be used in the text as abbreviations for diphosphopyridine nucleotide and triphosphopyridine nucleotide respectively; the oxidized and reduced forms, where these must be specified, may be referred to as oxidized DPN, reduced DPN, etc., or by any logical system, which must be explained in the text.

Symbols. In general the *Biochemical Journal* follows the *Report by the Symbols Committee of the Royal Society on Symbols, Signs, and Abbreviations recommended for British Scientific Publications* (1951) in the edition published by the Chemical Society, with the reservations that (*a*) full points are used after abbreviations in lower-case letters for all units, (*b*) temperature on the centigrade scale is shown simply as °, not ° C, (*c*) the abbreviations 'hr.' and 'sec.' are used, and (*d*) μm and $\mu\mu$ are preferred to n and p as prefixes indicating $\times 10^{-9}$ and $\times 10^{-12}$ respectively. The following list includes some of the more frequently used symbols, and certain special symbols not included in that Report.

Symbols for purely mathematical constants and operators are printed in roman type, e.g. e, base of natural logarithms. Other symbols for quantities, as distinct from abbreviations, are printed in italic type, when they are not Greek, e.g. *d* (density); *E* (extinction or electrode potential); *k*, *K* (velocity and equilibrium constants). They are not followed by a full point except at the end of a sentence. Abbreviations for units which are based on proper names (e.g. ampere, volt) are designated by small capitals, e.g. A, V, except for röntgen unit (r.).

about	*not circa*
absolute	abs.
acceleration due to gravity	*g*
acetamido	*not* acetylamino
acetic acid	'glacial' unnecessary when undiluted acid is referred to
acetone-dried powder	*not* acetone powder
acetylcholine	*no* hyphen
Adams catalyst	
adenosine diphosphate	
adenosine triphosphatase	two words
adenosine triphosphate	*not* adenosine triphosphoric acid; the three phosphorus atoms are distinguished as α, β and γ, thus: adenosine–P^{α}–O–P^{β}–O–P^{γ}
adrenaline	
aetio (prefix)	roman, *no* hyphen
*alanine	Ala
albumin	
aliquot	*not used; substitute* sample, portion *or* fraction

* See p. 6.

alkali, *pl.* alkalis	
allo	italic, *no* hyphen
alternating current	a.c.
amino acid	*no* hyphen
ampere	A
ampoule	
analyse	other words from root λύω end in 'lyse'; *see also* ise, ize
analytical standard of purity	A.R.
aneurin	*not used*; *use* thiamine, *alternative permitted* vitamin B_1
Ångström unit	Å
anteiso	*see Rep. Progr. Chem.* (1955), **52**, 302
anticholinesterase	one word
approximately	approx. (*or use* about, *not c.* or *ca.*)
aqueous	aq.
†arabinose	Ara
*arginine	Arg
artifact	
aryl group (in formulae)	Ar
ascorbic acid	*alternative permitted* vitamin C
*asparagine	(Asp-NH_2)
*aspartic acid	Asp
atmosphere	atm.
atomic weight	at.wt.
axial ratio (of spheroid)	preferably expressed as a/b (semi-axis of rotation = a, semi-equatorial axis = b)
baker's yeast	
benzamido	*not* benzoylamino
benzene (C_6H_6)	*not* benzol; benzine *not used*
benzyloxycarbonyl ($C_6H_5 \cdot CH_2 \cdot O \cdot CO \cdot$)	*not* carbobenzoxy *or* carbobenzyloxy
bis	roman
‡bivalent	
body weight	body wt. in Methods section and tables
boiling point	*no* hyphen; b.p.
brei	Anglicized, *pl.* breis
brewer's yeast	
British anti-lewisite	BAL; 2:3-dimercapto-propanol
British Pharmacopoeia	B.P. (with date)
British Thermal Unit	B.Th.U.
Büchner	
*iso*butanol	*iso*Butanol at the beginning of a sentence
calciferol	*use* ergocalciferol, *alternative permitted* vitamin D_2
calculated	calc.
Calorie (large, kilogram-calorie)	kcal.
calorie (small, gram-calorie)	cal.
capric acid	*use* decanoic acid
caproic acid	*use* hexanoic acid
caproyl	*use* hexanoyl
capryl, caprinoyl	*use* decanoyl
caprylic acid	*use* octanoic acid
caprylyl, capryloyl	*use* octanoyl
carbobenzyloxy	*use* benzyloxycarbonyl
carcass, carcasses	
catalatic	of catalase-like activity
catalyse	

*† See p. 6.

‡ Latin prefixes will be used with words of Latin origin such as 'molecular' and '-valent', e.g. 'unimolecular', 'bivalent', 'ter-', 'quadri-', 'quinque(i)-', 'sexi-', etc.; *not* 'monomolecular', 'divalent', 'tri-', 'tetra-', 'penta-', 'hexa-', etc.

cellophan	
centi- (prefix, 10^{-2})	c
centimetre	cm.
centimetre gram second	c.g.s.
centrifugation	centrifuging *preferred*
cerebrospinal fluid	c.s.f.
choline	
cholinesterase	
circa	*not used*; *see* about
cis-	italic, hyphen
cocarboxylase	
coefficient	coeff.
coefficient of variation	standard deviation/mean value
coefficient of variation %	100 × standard deviation/mean value
coenzyme I	Co I (*see also* diphosphopyridine nucleotide) (see p. 8)
compare	cf.
concentrated	conc.
concentration	concn.
concentration (symbol, e.g. in specific rotation)	c
‡configuration	D-, L-, etc.
connexion	
constant	const.
constant, equilibrium	K
constant, velocity	k
convert into	
corrected (e.g. m.p., for emergent stem)	corr.
crystalline, crystallized	cryst.
cubic	cu. *or as* e.g. $mm.^3$
curie	c
cycles per second	cyc./sec.
cyclo	italic, *no* hyphen
*cysteic acid	(Cy-SO_3H)
*cysteine	(CySH)
*cystine	(Cy$_2$S$_2$)
cytochrome *c*	
decomposition (m.p.)	decomp.
degrees (temperature)	§° C, ° K, ° F
deoxy (prefix)	*not* desoxy
deoxyribonucleic acid	*preferred to* deoxyribose nucleic acid
†deoxyribose	deoxyRib
dextrorotatory (as prefix) (*see also* configuration)	*dextro*, (+)-
dialysable	*not permitted*; *use* diffusible
dialysate	*for* diffusible material *use* diffusate
dialyse	
diffusion coefficient	D, $D_{20,w}$, D^0 etc. (*as for* sedimentation coefficient)
di-iodotyrosine	*use* hyphen (general rule: hyphen separates two similar vowels)
dilute	dil.
dioxan	
diphosphopyridine nucleotide	DPN (*see* coenzyme I) (see p. 8)
direct current	d.c.
dissociation constant, minus log of	pK
disulphide group	*alternative permitted* S·S group
dithionite (sodium)	$Na_2S_2O_4$, *not* hydrosulphite, hyposulphite

*† See p. 6. ‡ See p. 7.

§ Centigrade scale assumed in absence of indication; C to be inserted only when more than one scale is used.

electrode potential	E
electrode potential, standard	E_0
electrode potential, standard at constant pH	E'_0
electromotive force	e.m.f.
electron micrograph	*not* electron microgram
electron volt	ev
end point	
enzymic	*not* enzymatic
equation	eqn.
equivalent (weight)	equiv.
ethanol, ethanolic	*not* ethyl alcohol, *not* alcoholic
ethylenediaminetetra-acetic acid	EDTA *not* Versene
Experiment (with reference numeral)	Expt.; *pl.* Expts.
extinction (log I_0/I)	E $E^{1\%}_{1\,cm.}$ (specific extinction coefficient) $= E/cl$, where c is concn. % (w/v) and l is length in cm. ϵ (molecular extinction coefficient) $= E/cl$, where c is molar concn. and l is length in cm.
extracellular	*no* hyphen
farad	F
Faraday	F
Figure (with reference numeral)	Fig.; *pl.* Figs.
filter paper	
flavinadenine dinucleotide	
fluorimetry	measurement of intensity of fluorescence; *not* fluorometry
focusing	
foot	ft.
fractions, e.g. one-half, two-thirds	hyphen
freeze-dried	hyphen
freezing point	f.p.
frictional coefficient (molar)	f
frictional coefficient (molar) for sphere of same volume	f_0
†fructose	Fru
fuller's earth	
†galactose	Gal
gallon (Imperial)	gal.
gas constant per mole	R
gelatin	
†glucose	G
*glutamic acid	Glu
glutamine	(Glu-NH_2)
glutathione, oxidized	GSSG } see p. 8
glutathione, reduced	GSH } see p. 8
glycerophosphoric acid	glycerol phosphate (indicate position of substituent) *preferred to* phosphoglycerol; glyceryl phosphate and phosphoglyceryl *not allowed*
*glycine	Gly
glyoxaline	*preferred to* imidazole; *not* iminazole
gram	g.
gram-atom	g.atom
gram-calorie	cal.
gram-equivalent	g.equiv.
gram-molecule	g.mol. (*or* mole)
gravity, centrifugal	g, *preferred to* rev./min.
guinea pig	
haem, protohaem	prosthetic group of haemoglobin
haematin, protohaematin	oxidized haem

*† See p. 6.

haemochromogen	haem + base *or* haem + denatured protein
haemoglobinometry	
half-ester	hyphen
half-wave potential (polarography)	$E_{\frac{1}{2}}$
height	ht.
henry	H
hexose phosphate	two words
*histidine	His
hour	hr.
hydrogen-ion concentration, minus log of	pH, *pl.* pH values
hydrogen pressure (in atmospheres) in equilibrium with oxidation–reduction system, minus log of	rH
hydrolysate	
hydrolyse	
hydroquinone	*use* quinol
hydrosulphite, hyposulphite	*not used*; *see* dithionite
hydroxyl group	*alternatives permitted* OH group, hydroxy group
*hydroxylysine	Hylys
*hydroxyproline	Hypro
imidazole	glyoxaline *preferred* (*not* iminazole)
inch	in.
infrared	i.r.; one word, *no* hyphen
inhibitor constant	K_i (dissociation constant of inhibitor–enzyme complex)
insoluble	insol.
international unit	i.u.
iodometric	
ionic strength	I
ions: antimony, quinquivalent, Sb^{5+} antimony, tervalent, Sb^{3+} calcium, Ca^{2+} sulphate, SO_4^{2-}	
ise, ize	*use* termination 'ize' generally from Greek ending *-ίζειν*
isoelectric	
isoleucine	Ileu; iso *not* ital.
isomaltose	iso *not* ital.
joule	J
junior	jun.
kephalin	
keto acid	keto used only generically, *otherwise* oxo
keto sugars	*use* pentulose, hexulose, etc., *not* ketopentose, ketohexose, etc.
kilo- (prefix, $10^3\times$)	k
kilovolt-ampere	kVA
kilowatt-hour	kwh
Krebs–Ringer solution	reference to be given
laevorotatory (as prefix) (*see also* configuration)	*laevo*, (−)-
*leucine	Leu
light-absorption	hyphen
light petroleum	*not* petroleum ether: boiling range to be stated
lipid	*not* lipide, lipin, lipoid
litre	l.
logarithm, in text	log
logarithm (base 10), in formulae	log
logarithm (base e), in formulae	ln
Ltd.	*not* preceded by comma

* See p. 6.

lyse	spell with 's' *not* 'z'; derived words analogously (*see* analyse)
*lysine	Lys
†mannose	Man
maximum	max.
mecholyl	*use* acetyl-β-methylcholine
median effective dose	ED_{50}
median lethal dose	LD_{50}
melting point	m.p.
Messrs	*used* only for individuals (*not* corporate body) and then in acknowledgements only
meta (in names of organic compounds)	*m*-
metabolic quotients	*see* p. 4
methanol, methanolic	*not* methyl alcohol
methionine	Met
methyl orange, methyl red	lower case, *no* hyphen
metre	m.
Michaelis's constant	K_m (substrate concn. which gives half the saturation velocity). *See also* substrate constant
microanalysis microcrystalline micro-Kjeldahl micro-organism	general rule: *no* hyphen *except* before o *or* before proper name
micro- (prefix, $10^{-6}\times$)	μ
microgram	μg. (*not* γ)
microgram-atom	μg.atom (*not* μatom)
micromicro- (prefix, $10^{-12}\times$)	μμ (*preferred to* p)
micromilli- (prefix, $10^{-9}\times$)	μm (*preferred to* n)
micromole	μmole *not* μM
micron (10^{-6} metre)	μ
milli- (prefix, $10^{-3}\times$)	m
milliequivalent	m-equiv.
millilitre	ml.
millimicron (10^{-9} metre)	mμ
‡millimolar (concentration)	mM
millimole	m-mole *not* mM
minimum minute	min.
‡molar (concentration)	M
molecule, molecular	mol.
molecular proportion (in reactions)	mol.prop.
molecular weight	mol.wt.
multivalent	*not* polyvalent
mustard gas	di-(2-chloroethyl) sulphide *preferred*
namely	*not* viz.
nesslerize	
ninhydrin	
non-	hyphen
nor (in organic compounds)	roman, *not* italic, *no* hyphen
‡normal (concentration)	N
normal (in trivial names of organic compounds)	*n*-
normal temperature and pressure	*not* n.t.p.; *see* standard temperature and pressure
number (in enumerations)	no.
observed	obs.
ohm	Ω

*† See p. 6.

‡ Separated by a hyphen (and no full stop) from a chemical formula or name following it, e.g. M-NaCl; N-NaOH; N-sulphuric acid.

optical rotation	specific optical rotation (with concn. %, w/v), e.g. $[\alpha]_D^{20}$, $[\alpha]_{5461}^{25}$ molecular optical rotation ($=[\alpha] \times$ mol.wt./10), e.g. $[M]_D^{20}$, $[M]_{5461}^{25}$
optimum	*not* optimal
organophosphorus	*no* hyphen
ortho (in names of organic compounds)	*o*-
osmolar	the concentration producing an osmotic pressure equal to that of a molar solution of a perfect solute
ounce	oz.
ox	domestic bovine species without sexual distinction; *not* beef
oxaloacetic acid	
page, pages	p., pp.
para (in names of organic compounds)	*p*-
partial specific volume	$\bar{v}$
parts per million	p.p.m.
per (e.g. grams per litre)	/ (e.g. g./l.)
per cent	%
Petri dish	
phenyl glucuronide	
phenyl hydrogen sulphate	*not* phenol sulphate *or* phenyl sulphuric acid
*phenylalanine	Phe
phlorrhizin	
phosphoglyceryl	*not allowed*; *see* glycerophosphoric acid
phospholipid	*see* lipid
photomicrograph	
planchet	
potassium hydroxide	*not* caustic potash
potential difference	p.d.
pound (weight)	lb.
precipitate	ppt.
preparation	prep.
probability of an event's being due to chance alone	P
*proline	Pro
pyridoxine, pyridoxal	vitamin B_6 *permitted*
pyrrole	
quadrivalent	
quinol	*not* hydroquinone
quinquivalent	
radioactive	*no* hyphen
radioautograph	*not* autoradiograph *or* autoradiogram
reciprocal centimetre	$cm.^{-1}$
recrystallized	recryst.
refractive index	n. At stated temperature and wavelength represent as e.g. n_D^{20}
‡relative band speed (partition chromatography)	R, R_F
relative humidity	r.h.
respiratory quotient	R.Q.
revolutions	rev.
rev./min.	*not* r.p.m.; *use* **g** where possible
riboflavin	vitamin B_2 *permitted*
röntgen unit	r.
routinely	*not used*
sacrifice	*use* kill
schlieren	
second (time)	sec.
secondary (in names of organic compounds)	*sec.*-
sedimentation coefficient	S; *not* sedimentation constant

* See p. 6.

‡ See p. 3.

sedimentation coefficient corrected to 20° in water	$S_{20,w}$; S_{20} may be used, if unambiguous
sedimentation coefficient, at zero concentration	S^0, $S^0_{20,w}$ etc.
separating funnel	
*serine	Ser
sexivalent	
side arm	
sodium hydroxide	*not* caustic soda
soluble	sol.
solution	soln.
solvent systems	e.g. butanol–acetic acid–water (4:1:1, by vol.); butanol–acetic acid (4:1, v/v)
species (sing. and pl.)	sp., spp.
specific gravity	sp.gr.
specific heat	sp.ht.
square	sq. *or as* e.g. cm.^{2}
standard deviation	S.D.
standard error	S.E.
standard error of estimate of mean value	S.E.M.
standard temperature and pressure	s.t.p.
standard wire gauge	S.W.G.
steam-distil	
stilboestrol (3:4-di-*p*-hydroxyphenylhex-3-ene)	*give* systematic name first time; *not* called diethylstilboestrol
stoicheiometric	*not* stoichiometric
Student's *t* test	*no* literature reference
substituents (variable, in organic compounds)	R, R′, R″, *or* R^1, R^2, R^3, R^4 (if more than three)
substrate constant	K_s (dissociation constant of substrate–enzyme complex)
sulphydryl group	SH *or* thiol group *permitted*
‡sum	Σ *or* S
Svedberg unit (10^{13} × value in c.g.s. units)	s
tap water	
temperature	(abbrev.) temp.
tertiary (in names of organic compounds)	*tert.*-
tervalent	
test tube	
thiamine	*alternative permitted* vitamin B_1
thiophen	
*threonine	Thr
thyroxine	
time (symbol)	*t*
tocopherol	*alternative permitted* vitamin E
Tollens reagent	
toluene	*not* toluol
trans-	italic, hyphen
trichloroacetic acid	
1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)-ethane	DDT
triose phosphate	
tris (buffer)	chemical name, 2-amino-2-hydroxymethylpropane-1:3-diol, to be given at first mention
*tryptophan	Try; other amino acids have terminal 'e'
turnover number	of an enzyme; number of reaction cycles undergone by each active centre/min.
twofold, threefold, tenfold	for numbers above ten thus, 11-fold
*tyrosine	Tyr

* See p. 6.

‡ See notes on p. 4.

ultraviolet	u.v.
uncorrected (m.p., for emergent stem)	uncorr.
un-ionized	
univalent	
*valine	Val
value (e.g. iodine value)	val.
variety (e.g. botanical)	var.
velocity (symbol)	*v*
Versene (trade name)	*not used*; *use* ethylene-diaminetetra-acetic acid (-tetra-acetate)
viscometer	
viscosity (symbol)	η
volt	v
volume (abbrev. after number)	vol.
v/v	*used* only for two components; by vol. *used* for three or more components
water	*not* H_2O. Specify if not distilled
water bath	
watt	w
wavelength	λ
wavelength of *D* line of sodium (other wavelengths in Å)	D (as subscript)
wave number (unit)	$cm.^{-1}$
weight	wt.
X-ray	
χ^2 test	
†xylose	Xyl

*† See p. 6.

Additional copies may be obtained from the Editorial Office (*Biochemical Journal*), The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W.1, price 1*s.* 6*d.* post free.

The Preparation of [16-^{3}H]Progesterone

By W. H. PEARLMAN
Guy's Hospital Medical School, London, S.E. 1

(*Received* 16 *October* 1956)

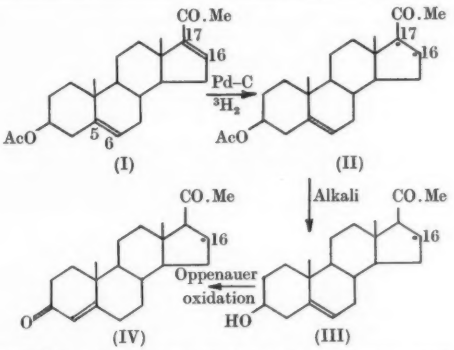
Radioactive progesterone of very high specific activity was required for certain *in vitro* experiments and at appropriate dilutions for *in vivo* studies on the metabolism of the hormone. It seemed likely that such a product might be prepared with comparative ease and economy by utilizing tritium as the isotopic labelling agent. Catalytic hydrogenation of an unsaturated steroid is a simple procedure and a large amount of hydrogen isotope can be introduced by this means. The double bond must be appropriately located in the molecule if the isotope is to be stably bound. Inasmuch as progesterone had been prepared from 3β-hydroxypregna-5:16-dien-20-one [commonly referred to as 16-dehydropregnenolone, unacetylated (I)] by differential catalytic hydrogenation, followed by Oppenauer oxidation (Marker *et al.* 1947), this approach to labelling progesterone with tritium appeared highly feasible, although only half of the tritium which might be initially introduced into ring *D* could be expected from theoretical considerations to be stably bound. The results obtained with the acetate of 16-dehydropregnenolone (I), which is commercially available and inexpensive, are described below.

EXPERIMENTAL

General. Melting points were determined on a Kofler-block type of apparatus and are corrected. *cyclo*Hexane, toluene and ether were stored over Na wire and redistilled over CaH_2 just before use. Light petroleum (AnalaR, b.p. below 40°) was treated with conc. H_2SO_4 in a separating funnel, carefully washed with water, dried over CaH_2 and distilled. The Al_2O_3 was Spence 'Type H' (Peter Spence, Widnes); it was treated with acetic acid, exhaustively washed with water and dried at 150° overnight.

Tritiation apparatus and procedure. The design of the apparatus was based on the one described by Berstein, Bennett & Fields (1952). The air in the system was displaced with N_2; tritium diluted with the theoretically required volume of H_2 was then introduced. The N_2 and H_2 were dried by passage over $Mg(ClO_4)_2$. A 10% Pd–charcoal catalyst was prepared by a hydrogenation procedure, as described by Fieser (1941); a magnetic stirrer afforded vigorous agitation of the reaction mixture which occupied about three-quarters of the reaction vessel. The progress of the reaction, which was carried out at atmospheric pressure and at room temperature, was followed with the aid of a mercury manometer, which had also served as a Toepler pump for the introduction of tritium into the system. When the reaction was over, the system was flushed with N_2; the entire process was carried out in a well-ventilated fume cupboard.

Determination of tritium. A weighed amount of steroid (containing about 0·03 μc of tritium) was dissolved in toluene (1·5 ml.) containing 0·5% (w/v) of the phosphor, *p*-terphenyl. The solution was transferred to an optical cup for measurement in a liquid scintillation counter (Avivi, Simpson, Tait & Whitehead, 1954) which had a detection efficiency for tritium of about 7%. A solution of a steroid of known specific activity was employed on each occasion as a reference standard; sufficient counts were recorded to ensure a standard deviation of $\pm 3\%$.



The sign • indicates localization of the isotope; a small amount of isotope may also be located at C-15, as discussed in the text.

Preparation of tritium-labelled steroids

[16:17-$^3H_2$]-3β-*Hydroxypregn-5-en-20-one acetate* (*II*). 3β-Hydroxypregna-5:16-dien-20-one acetate (I) (503 mg., 1·41 m-moles), m.p. 173–174°, was freed from any ethanol of crystallization before use by dissolving in *cyclo*hexane, evaporating to dryness *in vacuo*, and further drying in a vacuum desiccator over $CaCl_2$. The 5:16-diene (I) was redissolved in *cyclo*hexane (80 ml.), 10% Pd–charcoal catalyst (500 mg.) was added and the mixture stirred in a tritium–H_2 atmosphere (nominally 100 mc of tritium; H_2 was in slight excess of the theoretical requirement); the gas uptake ceased after 2 hr. (1·63 m-moles, corrected for the uptake by the catalyst; theory, 1·41 m-moles). The catalyst was removed by filtration and washed with ethanol. The combined filtrates were brought to dryness, and the residue, after three crystallizations from ethanol, gave a product (238 mg.), m.p. 150–151°; $[\alpha]_D^{22} + 13{\cdot}4 \pm 0{\cdot}8°$ in $CHCl_3$

(*c*, 5·01); specific activity 57·5 μc/mg. or 20·6 mc/m-mole. A second crop (106 mg.) of the same product was obtained on reworking the mother liquors. In all, the yield was 88%, representing a 20% radiochemical yield.

[16-^{3}H]-3β-*Hydroxypregn-5-en-20-one* (*III*). The [16:17-$^3H_2$]acetate (II) (226 mg.) was dissolved in a methanol solution (15 ml.) containing 5% (w/v) KOH and 20% (v/v) water. After refluxing for 3 hr., the solution was diluted with water and extracted with ether. The ether extract was washed with water and brought to dryness. The residue was crystallized twice from ethanol to yield a product (105 mg., 53%), m.p. 181–183°; specific activity 26·9 μc/mg. or 8·48 mc/m-mole. The percentage of tritium retained in the steroid molecule was 41.

[16-^{3}H]*Progesterone* (*IV*). [16-^{3}H]Pregnenolone (III) (104 mg.) was dissolved in dry toluene (50 ml.) and freshly distilled *cyclo*hexanone (7·5 ml.) was added. About 10 ml. of liquid was distilled to dry the system and then aluminium *iso*propoxide (300 mg.) was added. The reaction mixture was refluxed for 1 hr. and then steam-distilled, about 250 ml. of distillate being collected. The oily product was acidified with dil. HCl and extracted with ether. The ether extract was washed with 5% (w/v) Na_2CO_3 and finally with water. It gave on evaporation a partly crystalline product (119 mg.) which was purified by chromatography on Al_2O_3 (5 g.) with light petroleum–ether (3:1, v/v) as eluent. The crystalline product (94 mg.) was recrystallized from ether–light petroleum to afford prisms (57 mg., 55%), m.p. 128–129°; $[\alpha]_D^{23}$ +184±6° (*c*, 0·5); light-absorption max., 2400 Å (ϵ 16 650); specific activity 28·8 μc/mg. or 9·06 mc/m-mole.

The series of reactions were repeated but starting with 168 mg. (0·47 m-mole) of acetate (I) and 2·0 c (nominal) of tritium; [16-^{3}H]progesterone was obtained with specific activity 1·60 mc/mg. or 503 mc/m-mole (expected 1·73 mc/mg. on the basis of the results obtained in the previous run).

DISCUSSION

About one-fifth of the available tritium was incorporated into the steroid molecule in the initial step (catalytic reduction of I). To account for this discrepancy, the following factors, among others, might be mentioned: (1) catalytic exchange of hydrogen atoms between the gas phase and the solvent; (2) the relative roles of the gas phase and the solvent as donors of hydrogen atoms to the unsaturated steroid substrate; (3) the difference in the rates of reaction of hydrogen ($^1H_2$) and tritium which are ascribed to the difference in the effective mass of the isotope and hence of its 'zero point' energy; this may lead in certain instances to isotope fractionation (discussed by Kamen, 1951). The first factor may be the most important, since Farkas & Farkas (1939) had demonstrated a catalytic exchange of hydrogen atoms between molecular deuterium and the non-polar solvent, *cyclo*hexane. However, this exchange is very slow by comparison with that which obtains with hydroxylic solvents. The extent to which tritium may have entered into the *cyclo*hexane molecule was not determined in the present instance; the experimental conditions were not strictly comparable with those described by Farkas & Farkas (1939), who employed a platinized platinum foil and a hydrogen isotope of smaller mass. Incidentally, it should be noted that the acetate (I) was employed rather than the free hydroxyl compound, because catalytic exchange with this group would be expected to occur readily; also, that the 5:6 double bond resists reduction even in the presence of excess of hydrogen with palladium charcoal as catalyst.

Catalytic reduction of a double bond with hydrogen isotopes does not necessarily result in the localization of the isotope on the two carbon atoms. For example, Fukushima & Gallagher (1955) found that on catalytic reduction with deuterium of the 5:6 double bond of cholesterol acetate, about one-sixth of the total amount of deuterium introduced into the steroid molecule was located at C-7 and the remaining five-sixths equally distributed between C-5 and C-6. To account for this distribution of isotope, a mechanism was proposed involving the formation of a complex between the catalyst and the steroid molecule at C-5 and C-6 and its subsequent displacement toward the adjacent carbon, C-7. In view of these observations, it is conceivable that catalytic reduction of the 16:17 double bond of (I) may have led to the introduction of a small amount of tritium at C-15 with the major amount distributed between C-17 and C-16. Tritium at C-17 was not expected to be stably bound because C-20 ketones are known to undergo enolization, probably with the formation of a 17:20 double bond. Thus, Butenandt & Fleischer (1937) observed that 3β-hydroxypregn-5-en-20-one can be isomerized in part at the asymmetric centre (C-17) adjacent to the carbonyl group at C-20 by the action of 5% methanolic potassium hydroxide; the reactions are reversible and equilibrium is reached at a ratio of 70 parts of the normal ketone (C 17-β) to 30 parts of the 17-*iso*ketone (C-17α). In the present work, prolonged refluxing of the [16:17-^{3}H]acetate (II) with alkali resulted in a loss of 59% of the isotope content of the molecule.

It may be possible to prepare [16-^{3}H]progesterone of specific activity even higher than that described by utilizing 97% tritium gas and a few milligrams of the starting steroid (I); a micro-tritiation apparatus on a 1 ml. scale has been described by Glascock (1954).

Other methods for labelling progesterone with hydrogen isotope have been described. Koechlin, Kritchevsky & Gallagher (1950) prepared [11:12-$^2H_2$]-progesterone from methyl [11:12-$^2H_2$]lithocholate; the latter was obtained on reduction of methyl 3-acetoxy-11:12-cholenate in $CH_3 \cdot CO_2{}^2H$ with deuterium gas in the presence of PtO_2,H_2O. One should be able to prepare similarly the tritium-labelled hormone, but the radiochemical yield

from a limited amount of isotope would probably be low, since the introduction of isotope is made at the beginning of a long series of chemical transformations and also because isotopic labelling of the solvent employed in the catalytic reduction is required. This investigator also considered introducing the isotope into ring *C* of an appropriate unsaturated C_{21} steroid. Accordingly, pregn-9-ene-3:20-dione was prepared from 11α-hydroxyprogesterone (Peterson & Murray, 1952) according to procedures described by Mancera, Ringold, Djerassi, Rosenkranz & Sondheimer (1953) and Rosenkranz, Mancera & Sondheimer (1954), but the isolated 9:11 double bond resisted hydrogenation with *cyclo*hexane as solvent and palladium–charcoal as catalyst. Further experimentation along these lines was halted in favour of work on preparing [16-^{3}H]progesterone as described above. Another approach, requiring isotope-exchange procedure, had also been considered, but this was not promising since Fukushima & Gallagher (1952) could thereby introduce very little stably bound deuterium into the progesterone molecule, although they obtained better results with certain other C_{21} steroids: the amounts of stably bound deuterium or tritium incorporated were small, but still useful for metabolism experiments. It might be pointed out that exchange procedures are inherently inefficient in the utilization of isotope (although the degree of incorporation may be very high indeed), which is not as great an economic consideration in the case of deuterium as it is with tritium.

The potential usefulness of hydrogen isotope as auxiliary tracers for carbon and their limitations must be considered in each particular case (an excellent discussion is given by Kamen, 1951). In the author's experience (unpublished experiments) [16-^{3}H]progesterone has proved very useful in studying the intermediary metabolism of the hormone, not involving loss of the C-17 side chain; the isotope would probably be lost in that event.

SUMMARY

A comparatively simple and inexpensive route for the preparation of [16-^{3}H]progesterone of high specific activity (0·5 c/m-mole) is described.

The author gratefully acknowledges a personal grant from the Medical Research Council, and he wishes to express his appreciation of the hospitality afforded him by members of the staff of Guy's Hospital Medical School.

Mrs M. C. Bullas made the tritium analyses in the Barnato Joel Laboratories of The Middlesex Hospital Medical School, London, for which the author is indebted. Mr N. Veall, of the radioisotopes division at Guy's Hospital Medical School, gave invaluable advice. Dr D. H. Peterson, The Upjohn Company, very kindly furnished 11α-hydroxyprogesterone.

REFERENCES

Avivi, P., Simpson, S. A., Tait, J. F. & Whitehead, J. K. (1954). *2nd Radioisotope Conf.* vol. 1, p. 313. London: Butterworths Scientific Publications.

Berstein, I. A., Bennett, W. & Fields, M. (1952). *J. Amer. chem. Soc.* **74**, 5763.

Butenandt, A. & Fleischer, G. (1937). *Ber. dtsch. chem. Ges.* **70**, 96.

Farkas, A. & Farkas, L. (1939). *Trans. Faraday Soc.* **35**, 917.

Fieser, L. F. (1941). *Experiments in Organic Chemistry*, 2nd ed., p. 459. Boston: D. C. Heath and Co.

Fukushima, D. K. & Gallagher, T. F. (1952). *J. biol. Chem.* **198**, 871.

Fukushima, D. K. & Gallagher, T. F. (1955). *J. Amer. chem. Soc.* **77**, 139.

Glascock, R. F. (1954). *Isotopic Gas Analysis for Biochemists*, p. 222. New York: Academic Press.

Kamen, M. D. (1951). *Radioactive Tracers in Biology*, 2nd ed., ch. 7. New York: Academic Press.

Koechlin, B. A., Kritchevsky, T. H. & Gallagher, T. F. (1950). *J. biol. Chem.* **184**, 393.

Mancera, O., Ringold, H. J., Djerassi, C., Rosenkranz, G. & Sondheimer, F. (1953). *J. Amer. chem. Soc.* **75**, 1286.

Marker, R. E., Wagner, R. B., Ulshafer, P. R. Wittbecker, E. L., Goldsmith, D. P. J. & Ruof, C. H. (1947). *J. Amer. chem. Soc.* **69**, 2167.

Peterson, D. H. & Murray, H. C. (1952). *J. Amer. chem. Soc.* **74**, 1871.

Rosenkranz, G., Mancera, O. & Sondheimer, F. (1954). *J. Amer. chem. Soc.* **76**, 2227.

The Synthesis of Serum Albumin and Tissue Proteins in Slices of Rat Liver and Liver Tumour

By P. N. CAMPBELL and NANCY E. STONE

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

(*Received* 3 *September* 1956)

There have been many attempts in the past to compare the way in which proteins are synthesized in normal and tumour tissue. However, for the most part the comparison has been made between the synthesis of the complex mixtures of proteins contained in the two types of tissue. Zamecnik (1952) has pointed out in his review how limited is the value of such comparisons. If the biosynthesis of proteins in tumour tissue does in fact differ from that in normal tissue, then such differences are more likely to be brought to light by comparing the synthesis of a particular protein in the two types of

tissue rather than by comparing the synthesis of mixtures of proteins. The present paper describes a system in which such a comparison might be made.

Peters & Anfinsen (1950*b*) demonstrated the synthesis of serum albumin in chick-liver slices. Later Miller, Bly, Watson & Bale (1951), using perfused rat liver, showed that the liver was the main, if not the only, source of serum albumin. The synthesis of albumin must, therefore, be considered a characteristic function of the liver cell. If liver cells retain this function during the transformation to malignancy, then it should be possible to study the synthesis of serum albumin both in liver and tumour.

The method of Peters & Anfinsen (1950*b*), in which the amount of albumin present in slices during the course of incubation is determined by means of a specific antiserum, has been adapted to the rat. This animal was chosen owing to the facility with which liver tumours can be induced by feeding with 4-dimethylaminoazobenzene. By including a radioactive amino acid in the medium in which the slices are incubated, not only is it possible to follow the changes in the amount of albumin present, but the rate of incorporation of amino acid into this protein may also be studied. Thus two criteria of protein synthesis are available.

The results of such experiments show that rat-liver slices synthesize serum albumin and that slices of liver tumour also possess this property. Experiments are described which indicate that the albumin is being synthesized by the malignant cells in the tumour slices and that the synthesis is not due to contamination of the slices with normal liver cells.

The incorporation of radioactive carbon from [^{14}C]glucose into the tissue proteins was studied with rat liver and liver-tumour slices by Zamecnik, Loftfield, Stephenson & Steele (1951). Later Campbell (1955) showed that the conversion of [^{14}C]glucose into radioactive protein was more efficient in liver-tumour slices than in liver slices. In view of these results the synthesis of serum albumin by liver and liver-tumour slices has also been studied in the presence of [^{14}C]glucose.

Preliminary accounts of some aspects of this work have already been reported (Campbell & Stone, 1955, 1956).

MATERIALS AND METHODS

Animals and production of tumours. Liver tumours were induced in male Wistar albino rats highly inbred at this Institute, by feeding on a diet containing 0·06% of 4-dimethylaminoazobenzene under the conditions previously described (Campbell, 1955).

When a group of ten animals was fed on a diet in which 3′-methyl-4-dimethylaminoazobenzene (Price, Harman, Miller & Miller, 1952) replaced 4-dimethylaminoazobenzene, fewer rats developed tumours, and in the affected livers the tumours were less discrete.

Histological examination. Pieces of liver and tumour were fixed in 0·9% NaCl containing 4% of formaldehyde, sectioned and stained with haematoxylin. To show the presence of glycogen, carmine stain was used. In several experiments, the tissues were examined by frozen section.

Radioactive compounds. Uniformly labelled D-[^{14}C]-glucose (100 μC$\equiv$1·64 mg.), [^{14}C]glycine (100 μC$\equiv$8·6 mg.) and L-[^{14}C]lysine (100 μC$\equiv$2·5 mg.) were supplied by the Radiochemical Centre, Amersham.

Preparation of rat-serum albumin

The method of Keltz & Mehl (1954), based on Cohn's method 10 (Cohn *et al.* 1946), could not be repeated successfully. Paper electrophoresis in veronal buffer, pH 8·6, showed that albumin was present in the early precipitates, and that the final albumin fraction was contaminated with an appreciable quantity of globulin. When the method of Ulrich, Li & Tarver (1954) was used, a greater yield of crude albumin was obtained, but it too contained a high proportion of globulin impurities.

The following method for the preparation of albumin was, therefore, adopted.

Serum was obtained from male rats by heart puncture. The serum was fractionated by the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution to the serum diluted (1:1) with 0·9% NaCl. The precipitate was removed by filtration, and after dialysis the filtrate was freeze-dried. From 52 ml. of blood 886 mg. of crude albumin was obtained, which was further fractionated by zone electrophoresis.

Zone electrophoresis. The method first described by Flodin & Porath (1954), in which electrophoresis is carried out in columns which are subsequently eluted with buffer, was used. Details of the apparatus were as described by Porath (1954) except that the sintered-glass plate at the bottom of the column was replaced by a small pad of glass wool.

Partially acetylated cellulose, which was used to pack the column (54 cm. $\times$ 3 cm.), was prepared as follows. Absorbent cotton wool (300 g.), broken into small pieces, was refluxed for 20 hr. with 375 ml. of acetyl chloride and 4875 ml. of ethanol. The finely divided product was filtered off and washed first with ethanol and then with water. The larger particles were removed by flotation.

Electrophoresis was carried out in a borate–phosphate buffer (0·0115 M-$Na_2B_4O_7$–0·0155 M-NaH_2PO_4; I=0·05. pH 8·6). After equilibration of the cellulose column with buffer, crude albumin (300 mg.) dissolved in 2 ml. of buffer was added to the column and washed 2–3 cm. into the cellulose. A current of 30 mA was applied for 42 hr. The protein was then eluted with the same buffer, at a rate of 10–15 ml./hr.; 2·5 ml. fractions were collected. The optical density of the fractions at 280 mμ was determined in a spectrophotometer with 0·5 cm. cells. The fractions containing the albumin peak were combined, dialysed and freeze-dried, yielding 213 mg. of albumin.

Antiserum against rat-serum albumin. Rabbits were injected with alum-precipitated albumin, as described by Campbell (1955). The amount of antibody in the rabbit serum was estimated by serial dilution to be at least 10 mg./ml. of serum.

Immunological examination. Immunological examinations of purified rat-serum albumin and of liver and tumour extracts, for antigenic heterogeneity, were carried out by

the combined electrophoresis and agar-gel diffusion technique of Grabar & Williams (1955). The antigen–antiserum reaction was also studied by the method of Oudin (1948).

Radioactive rat-serum albumin. A male rat (340 g.) was injected intraperitoneally with 50 μc (1·25 mg.) of L-[^{14}C]lysine in 0·5 ml. of 0·9% NaCl. After 6 hr. without food, but with water, the animal was injected intravenously with heparin and bled from the jugular vein, while the vena cava was perfused with saline. The blood–saline mixture was centrifuged and albumin obtained from the supernatant by fractionation as described above. The yield of albumin was 233 mg.

Incubation of slices and estimation of serum albumin

Incubation procedure. Slices of tissue were cut by hand and suspended in the bicarbonate medium described by Peters & Anfinsen (1950*b*). Outside slices of tissue were rejected and tumours, discrete and usually 0·5–2 cm. in diameter, were trimmed to remove unaffected liver before slicing.

To reduce the weight of albumin initially present, 4–5 g. of slices was washed in 100 ml. of medium by gentle agitation for 45 min. at room temperature. The supernatant was decanted, 50 ml. of fresh medium added, and washing resumed for a further 15 min. The slices were drained on filter paper, divided into approximately equal batches (0·5–2 g., depending on the experiment), and weighed and incubated in 125 ml. flasks containing 10 ml. of medium and the appropriate amount of radioactive compound at 37° in an atmosphere of $O_2 + CO_2$ (95:5). The flasks were shaken 100 times/min. during incubation. Kidney slices contained so little albumin that this tissue was not washed before incubation.

After incubation each flask was cooled in an ice bath and the contents were homogenized in a Potter-type all-glass homogenizer for 2 min. at 4500 rev./min. at room temperature. The pestle was tightly fitting. Care was taken to keep the procedure as uniform as possible. The tissue suspension was centrifuged at 15 000 *g* for 20 min. at 2°, the supernatant drawn off and diluted with an equal volume of 0·9% NaCl containing 1:10 000 Thiomersalate (sodium ethylmercurithiosalicylate). This solution, referred to throughout as a tissue extract, was stored at −20°.

Estimation of serum albumin in tissue extracts. The method of Peters & Anfinsen (1950*b*), as described in detail by Campbell (1955), was followed. Before the estimations, tissue extracts were spun at 15 000 *g* for 20 min. at 17°, to remove material precipitated by freezing and subsequent thawing. In some experiments it was necessary to modify the procedure as follows.

Separation of slices and medium before homogenizing. After incubating and cooling in an ice bath the mixture of slices and medium was centrifuged at 10 000 *g* at 0° for 8 min. The supernatant was drawn off and diluted with an equal volume of 0·9% NaCl containing 1:10 000 Thiomersalate. The slices were homogenized in 5 ml. of ice-cold bicarbonate medium, and the homogenate was treated as described in the previous section.

Clearing extracts with chick-serum albumin–antiserum precipitates. Chick-serum albumin and the specific antiserum to this protein were prepared as described by Campbell (1955). Tissue extract (2 ml.) was incubated for 30 min. at 37° with 0·4 ml. of antiserum solution (10%, w/v) and 0·6 ml. of chick-serum albumin (0·1%). After storing overnight at 2°, the precipitate was spun off at 2° for 20 min. at 15 000 *g*, and the supernatant, now clear, used for estimation of albumin. The validity of this procedure is discussed later.

Measurement of radioactivity

Protein. Proteins were precipitated from tissue extracts by addition of an equal volume of 10% trichloroacetic acid. Non-protein material was removed as described by Zamecnik *et al.* (1951). The dried protein was plated on 0·28 cm.^{2} Perspex disks and counted at infinite thickness in a Geiger–Müller counter with a mica end-window.

Albumin. The radioactivity of the serum albumin in the tissue extracts was determined on the albumin–antiserum precipitates counted at infinite thinness on 2 cm.^{2} polythene disks as described in the Results section.

Glycogen and free-sugar estimations. In certain experiments the glycogen content of liver and tumour was estimated as follows. Weighed samples of liver and tumour slices (0·3–0·5 g.) (unwashed) were homogenized in 10 ml. of 5% trichloroacetic acid and glycogen was estimated according to the method of Kahan (1953), except that the modified anthrone reagent of Fairbairn (1953) was used. Free sugars were estimated in the solution in trichloroacetic acid (after treatment with $ZnSO_4$ and $Ba(OH)_2$) by the method of Hagedorn & Jensen (1923*a*, *b*).

RESULTS

Cytology of liver tumours

Rats which had been fed on the diet containing 4-dimethylaminoazobenzene showed considerable variation in the proportion of animals affected and in the gross appearance of the tumours. For this reason the tissue for any one experiment was generally taken from three or more animals, the slices being mixed before incubation. On several occasions liver slices from normal rats were studied, and this tissue will be referred to as 'normal liver', to distinguish it from the unaffected liver of tumour-bearing animals, which it is convenient to designate 'liver'.

Histological examination of the liver tumours showed that two types of malignant cells predominated. The first, hepatoma cells, are derived from liver cells, and the second, cholangioma cells, from the cells of the bile ducts. Some tumours contain a mixture of the two cell-types; others consist primarily of one type only. The only way of distinguishing hepatomas and cholangiomas is by microscopic examination. Since this could not be performed in every case, the malignant tissue used in most experiments probably contained both types of cells, and has therefore been referred to as 'tumour' tissue. In experiments in which the type of malignant cells predominating is specified, the tumours were classified by histological examination of frozen sections.

Histology of the tumours used for slicing showed that the amount of normal liver tissue present was only a small proportion of the whole. Nevertheless, it appeared desirable to devise a method of estimating the extent of the contamination of the tumour slices by normal liver tissue.

Edwards & White (1941) and Zamecnik *et al.* (1951) have shown that the glycogen content of liver tumour is much lower than that of liver. Histological examination of the tumours used in the present experiments confirmed this. Glycogen estimation seemed, therefore, to provide a suitable criterion for the presence of normal liver tissue. In three separate experiments the glycogen content (expressed as % wet wt. of tissue) in liver was 4·7, 4·7, 1·8, and in tumour 0·16, 0, 0. These results suggested that contamination of tumour with normal cells was very small but do not exclude the possibility that liver cells lying adjacent to tumour cells might have a very low content of glycogen. By selecting sections for histology which contained tumour and liver tissue it was possible to show that this was not so. It seemed unlikely, therefore, that the presence of liver cells in the tumour slices would complicate the interpretation of results obtained with such slices.

Rat-serum albumin

Fig. 1 shows the diagram obtained when the crude albumin fraction obtained by ammonium sulphate precipitation was subjected to electrophoresis on a small column of cellulose. The two smaller peaks are presumed to be α- and β-globulins. For comparison, Fig. 2 shows the diagram obtained when the albumin fraction obtained by alcohol fractionation according to the method of Ulrich *et al.* (1954) was subjected to electrophoresis under identical conditions. It will be seen that the preparation obtained with ammonium sulphate contains relatively less globulin than that obtained with ethanol, and since it was the simpler procedure it was adopted.

The crude albumin preparation was further fractionated on a cellulose column. The protein from the albumin peak was tested for homogeneity by electrophoresis in a Tiselius apparatus in a veronal buffer, pH 8·6, and in acetate buffer, pH 4·5. The results shown in Fig. 3 indicate that the albumin contained a small amount of globulin but there was no clear indication of a split in the albumin peak, such as was reported by Hoch-Ligeti, Hoch & Goodall (1949).

This albumin, which was used to prepare the antiserum, was further tested for heterogeneity by the agar-gel diffusion technique of Oudin (1948). This showed that it contained small quantities of a second component.

The combined electrophoretic and diffusion method of Grabar & Williams (1955) was also used to determine the composition of the albumin preparation. Thus in veronal buffer, pH 8·6, small

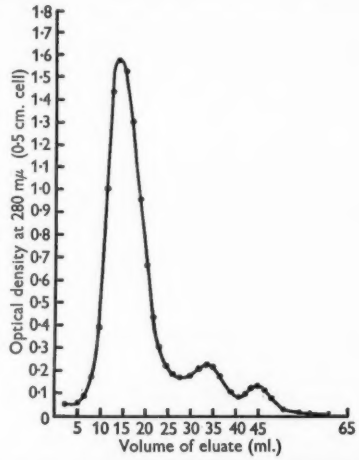


Fig. 1. Zone electrophoresis of rat-serum albumin obtained from whole serum after removal of the precipitate formed with 0·5-saturated $(NH_4)_2SO_4$. Protein (50 mg.) was added to the column (46 cm. × 1·5 cm.) packed with 'cellulose'. A current of 10 mA (500 v) was applied for 24 hr. Borate–phosphate buffer (see Methods section) was used.

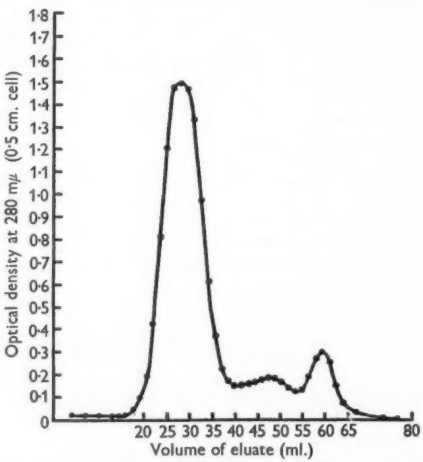


Fig. 2. Zone electrophoresis of rat-serum albumin obtained from whole serum by ethanol fractionation (see Methods section). The conditions were identical with Fig. 1.

quantities of a second component with a lower electrophoretic mobility than that of albumin were detected (see Fig. 4).

Specificity of antiserum

To test whether cross-reactions occurred in the tissue extracts between the antiserum and proteins other than albumin, liver and tumour extracts were examined by the immuno-electrophoresis technique. Since extracts which had been concentrated by freeze-drying failed to produce any precipitation with antiserum on the plates, presumably due to their low content of albumin, ammonium sulphate fractionation was used to concentrate the albumin in the extracts. The results, shown in Fig. 4, indicate that the antiserum reacted with only one component in the tissue extracts, which has an electrophoretic mobility similar to serum albumin. This is good evidence that the antiserum specifically precipitates the albumin in the tissue extracts and that the proteins precipitated in the extracts of liver and tumour are identical.

A similar experiment to that illustrated in Fig. 4 was carried out with fresh serum and purified albumin. Since the results were identical in each case it is concluded that the antiserum reacted with the same components in the fresh serum as in the purified albumin and did not cross-react with other proteins in the serum.

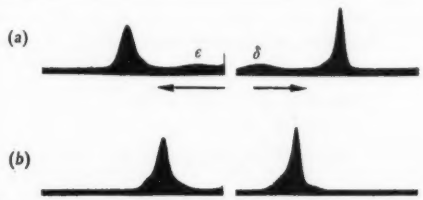


Fig. 3. Electrophoretic patterns of purified rat-serum albumin used for the preparation of antiserum. Each arrow indicates the position of the boundary at the start of the run and the direction of movement. Potential gradient, 4·5 v/cm. $I = 0{\cdot}1$, 1% protein. (*a*) Sodium veronal buffer, pH 8·59. Duration of run, 3 hr. 30 min. (*b*) Sodium acetate buffer, pH 4·52. Duration of run, 3 hr. 35 min.

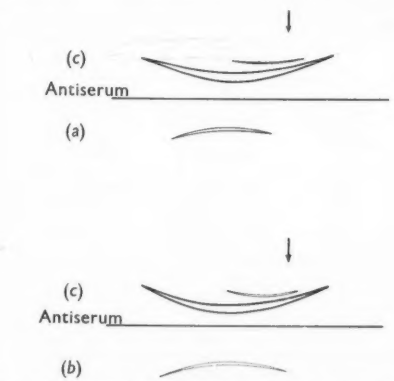


Fig. 4. Immuno-electrophoretic patterns of fractions of (*a*) liver and (*b*) tumour extracts compared with purified rat-serum albumin (*c*). Protein (2·5 mg. as 6% solution) was applied at starting position indicated by arrow, and current (40 mA, 150 v, for two agar plates arranged in parallel) applied for 6 hr., with sodium veronal buffer, pH 8·2, $I = 0{\cdot}05$. Antiserum (5%) was added to centre well and plates were stored for 14 days at 2° before photographing. (Diagrammatic representation.)

Estimation of rat-serum albumin in tissue extracts

Preparation of standard curve. When a standard solution of rat-serum albumin was assayed by the antiserum method the standard curve was very similar to that obtained with chick-serum albumin (see Campbell, 1955). Since small differences in incubation and centrifuging procedures were liable to affect the result slightly, a fresh standard curve was prepared for each assay. Duplicate estimations of albumin usually agreed to within ±10% of the mean, although solutions very low in albumin could not always be assayed with such accuracy. On these occasions it was observed that the albumin–antiserum precipitate from the tube containing less albumin was disproportionately greater than the precipitate in the second tube.

Treatment of extracts with chick-serum albumin and antiserum. When attempts were made to estimate the amount of albumin in tissue extracts by the addition of antiserum, followed by estimation of the amount of precipitate, the results were rather variable. This seemed to be due in part to the presence of substances which were precipitated with the albumin–antiserum precipitate but which failed to dissolve in 0·1 M-sodium carbonate. Freezing and thawing the extracts did not consistently improve the results, nor did starving the animals overnight before taking the livers and tumours.

Askonas, Campbell, Humphrey & Work (1954) cleared whey-protein solutions by carrying out an ovalbumin–antiovalbumin precipitation in the extracts; the interfering substances were adsorbed on to the precipitate, which was removed. Chick-serum albumin and its specific antiserum were added to the tissue extracts in the present experiments. The effect of this treatment on the estimation of serum albumin in tissue extracts can be seen from Table 1 (Expts. VII and XI), in which the apparent level of albumin was appreciably lowered by the clearing process. This was most marked in liver-

tissue extracts, which before clearing were invariably more clouded than tumour extracts. Once the interfering material had been removed, albumin estimations were more readily reproducible. However, it is of interest to note that percentage change in albumin during incubation of slices was not greatly altered by clearing. For the latter reason it has been thought worth while to include among the results some estimations of albumin made on uncleared solutions, since these reflect changes in albumin content, if not absolute amounts present in different extracts.

The question arose whether clearing tissue extracts by a precipitation between chick-serum albumin and its specific antibody caused loss of rat-serum albumin, either by adsorption or by a cross-reaction with added antibody. The latter possibility was tested by incubating both standard solutions of rat-serum albumin and liver and tumour extracts with antiserum to chick-serum albumin. In no case was any trace of precipitate detected. The remaining possibility, that rat-serum albumin might be lost by adsorption, was tested by adding radioactive rat-serum albumin to an extract of normal liver and clearing this extract with chick albumin–antiserum. Since the chick albumin–antiserum precipitate was not radioactive it is clear that rat albumin had not been co-precipitated. In the experiments in which slices were incubated with radioactive amino acid and the extracts were cleared with chick albumin–antiserum the radioactivity of the rat albumin was not much affected by the process of clearing (Table 3). However, since the chick albumin–antiserum precipitate was significantly radioactive, the clearing of extracts is presumed to be due to adsorption.

Completeness of precipitation. To test for the presence of soluble albumin–antiserum complexes in the tissue extracts to which antiserum had been added, radioactive albumin (50 μg.) was incubated in the usual way with antiserum (3 mg.). The precipitate was spun off, and the supernatant dialysed, freeze-dried and 24 mg. of the product counted at infinite thickness on a 0·28 cm.^{2} disk. There was no detectable increase in counts above the background. This indicated that all the albumin had been precipitated by reaction with antiserum.

Estimation of specific radioactivity of albumin–antiserum precipitates

In spite of the fact that precipitation of albumin with antiserum increased the amount of protein available for counting by at least ten times, the amount of albumin likely to be present in tissue extracts was so low that counting at infinite thickness was impossible. Albumin was precipitated from tissue extracts under the conditions described for the estimation of albumin in such extracts. The

Table 1. *Synthesis of albumin in slices of liver from tumour-bearing rats and in slices of liver tumours*

Results are given in mg. of albumin/g. of wet tissue; percentage increase is shown in parentheses. Extracts were not cleared with chick albumin–antiserum precipitate except where stated.

Expt. no.	Liver			Tumour		
	0 hr.	2 hr.	4 hr.	0 hr.	2 hr.	4 hr.
I	1·98	2·82 (43)	2·77 (40)	0·74	1·12 (52)	1·35 (84)
III	1·05	1·87 (78)	2·63 (150)	0·55	0·74 (34)	1·19 (115)
VII	1·50	1·82 (21)	1·82 (21)	0·41	0·68 (66)	0·70 (71)
VII*	0·92	1·08 (18)	1·08 (18)	—	—	—
XI	1·29	2·22 (72)	2·34 (82)	0·49	0·69 (40)	0·88 (78)
XI*	0·70	1·09 (56)	1·35 (94)	0·43	0·62 (46)	0·76 (78)

* The extracts were cleared with chick albumin–antiserum precipitate before estimation of albumin.

Table 2. *Effect of amount of tissue on uptake of ^{14}C from a fixed glycine pool*

Different weights of liver and tumour slices were incubated with a constant amount of [^{14}C]glycine, and the radioactivity of albumin and TCA proteins was determined. In two separate experiments, A and B, slices were incubated for 2 hr. at 37° with 1·5 μc of [^{14}C]glycine (130 μg.). The results are expressed in counts/min./cm.^{2} at infinite thickness.

Wt. of tissue (g.)	TCA protein				Albumin			
	Expt. A		Expt. B		Expt. A		Expt. B	
	Liver	Tumour	Liver	Tumour	Liver	Tumour	Liver	Tumour
0·5	1327	2325	2813	4966	2088	452	3208	1720
1·0	1502	2209	2481	4716	2343	592	2250	1316
1·5	1414	1777	1839	3313	1972	383	1821	1022
2·0	1387	1262	1817	3159	2320	406	1656	726

protein was dissolved in 5N-NH_3 soln. and added in amounts between 100 and 700 μg. to disks (2 cm.^{2}). When dry these could be counted at infinite thinness, and the amount of protein on the disk determined afterwards by dissolving in 0·1M-sodium carbonate and measuring the optical density of the solution at 280 mμ.

Fig. 5 shows that amounts of protein up to 700 μg./2 cm.^{2} disk could be counted without requiring a correction for self-absorption. This was surprising, since 200 μg. of alanine was the maximum which could be counted at infinite thinness on a 2 cm.^{2} disk under the conditions described by Campbell (1955). The explanation appears to be that when a solution of amino acid is dried on a polythene disk the amino acid is deposited around the edge. Owing to the steep walls of the disk the counting efficiency is reduced. However, when a protein solution is dried on such a disk there is a more even distribution of protein over the whole disk and the counting efficiency is thereby increased. This is confirmed by the fact that the factor for conversion of the infinite thinness count/mg. of protein into infinite thickness on a 1 cm.^{2} disk is 4·8, whereas with an amino acid it is 7·6.

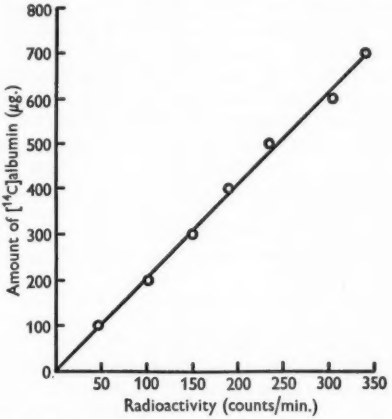


Fig. 5. Self-absorption curve of [^{14}C]-labelled albumin, for determination of specific radioactivity of albumin–antibody precipitates.

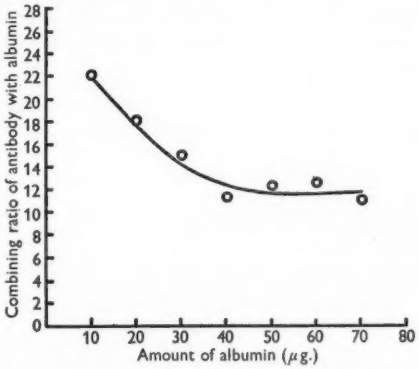


Fig. 6. Curve showing change in combining ratio of antibody with albumin. Combining ratios were calculated from the specific radioactivities of albumin–antibody precipitates obtained by treatment of various weights of serum albumin of known radioactivity with antiserum under standard conditions.

Combining ratios of rat-serum albumin with antibody. In order to determine whether the ratio of antibody to antigen varied when albumin was precipitated from solutions containing different concentrations of albumin, use was made of ^{14}C-labelled albumin. The specific activities of ^{14}C-labelled albumin–antibody complexes, prepared by adding different amounts of albumin (10–70 μg.) to a constant amount of antiserum (3 mg.) in solution, increased with increasing weight of added albumin until a constant figure was reached, when 50 μg. or more of albumin was used. Since the radioactivity of the albumin alone (475 counts/min./mg. at infinite thinness) was known, the weight of antibody combining with a fixed weight of albumin could be calculated. The results are shown in Fig. 6. Thus the ratio changed from 22·5 parts of antibody:1 part of albumin, at 10 μg. of added albumin, to 12:1, when 50 μg. of albumin was used.

Determination of specific radioactivity of albumin in tissue extracts. Owing to the variation in the combining ratio of antibody to albumin the radioactivity of the albumin in the different tissue extracts could not be directly derived from the specific radioactivity of the albumin–antibody complexes. Thus the concentration of albumin was first determined in a sample of the tissue extract. A further sample was then treated with antiserum and the specific activity of the albumin–antiserum precipitate was determined. Since the concentration of albumin in the extract was known, the combining ratio of albumin to antibody could be obtained from Fig. 6. Hence the specific radioactivity of the albumin in the extract could be calculated. In order to enable comparisons to be made the counts obtained at infinite thinness were converted to infinite thickness. Duplicate determinations of specific activity were within 10%.

Effect of amount of tissue on incorporation of [^{14}C]*glycine*

In order to determine the effect on the radioactivity of the tissue proteins of incubating different amounts of tissue slices with the same amount of radioactive amino acid, batches of tumour and liver

slices (0·5–2 g.) were incubated for 2 hr. in 10 ml. of medium containing the same amount of [^{14}C]glycine (1·5 μc, 130 μg.). Table 2 shows the results of two experiments in which the radioactivity of the proteins precipitated by trichloroacetic acid (TCA protein) and of serum albumin was determined. In Expt. A the radioactivity of both the TCA protein and the albumin remained relatively constant in liver, whereas in tumour the radioactivity of the TCA protein fell with increasing weight of tissue, and the albumins showed no definite trend. In Expt. B the radioactivity of both TCA protein and albumins in liver and tumour decreased with increasing weight of tissue.

Serum-albumin synthesis in liver and tumour slices

The results of estimations on the amount of albumin in liver and liver-tumour slices during incubation are shown in Table 1. In every case it will be seen that the amount of albumin increased during the incubation period, although there was considerable variation between the results of individual experiments. The results suggest that the albumin increase in the liver slices falls off more quickly than with tumour slices.

In Expt. VII, the effectiveness of washing liver slices before incubation was determined. Without washing, the level of albumin (in the uncleared extract) was 4·4 mg./g.; after 40 min. washing this had fallen to 2·37 mg./g., and at the end of 1 hr. the level was 1·5 mg./g.

The results of incorporation studies with [^{14}C]-glycine run parallel with the increases in albumin in tissue slices during incubation, for in every case the uptake of radioactivity by albumin increased with time. In some experiments incorporation of radioactivity into albumin was similar in the two tissues, whereas in others uptake by tumour albumin was lower than that by liver albumin. The results of a typical experiment are shown in Table 3. It will be observed that the radioactivity of the albumin is very much greater than that of the total soluble protein fraction of both liver and liver tumour.

Campbell (1955) showed that radioactivity from [^{14}C]glucose was more efficiently incorporated into the soluble protein of liver tumour than of liver when slices of these tissues were incubated with [^{14}C]glucose. These experiments have now been extended. Table 3 shows that not only is the soluble protein fraction from liver tumour very much more radioactive than a similar fraction from liver after incubation of slices with [^{14}C]glucose, but that the radioactivity of tumour albumin is also much greater than that of liver albumin.

Table 3. *Incorporation of radioactivity into albumin and TCA protein of liver and of tumour slices incubated with 2·5 μc of [^{14}C]glycine (Expt. XI, Table 1) or 4·5 μc of [^{14}C]glucose*

Radioactivity is expressed as counts/min./cm.^{2} at infinite thickness.

	Liver				Tumour			
Time of incubation (hr.)	... 2		4		2		4	
Precursor	Albumin	TCA protein	Albumin	TCA protein	Albumin	TCA protein	Albumin	TCA protein
[^{14}C]Glycine*	9072	2535	12 744	3613	6750	4512	11 502	6983
[^{14}C]Glycine†	8100	—	13 284	—	8208	—	11 556	—
[^{14}C]Glucose*	1404	63	1 620	111	7020	1128	9 126	1835

* Tissue extracts were not cleared with chick albumin–antiserum precipitate before precipitation of albumin.
† Extracts were cleared.

Table 4. *Synthesis of albumin and incorporation of radioactivity into albumin and soluble proteins of liver, hepatoma and cholangioma*

Slices were incubated with 2·5 μc of [^{14}C]glycine. Results are given in mg. of albumin/g. of wet tissue; percentage change is shown in parentheses. Tissue extracts were cleared before albumin was estimated or precipitated. Radioactivity is expressed as counts/min./cm.^{2} at infinite thickness.

Time of incubation (hr.)	... 0			4		
		Radioactivity			Radioactivity	
	Wt. of albumin (mg.)	Albumin	TCA protein	Wt. of albumin (mg.)	Albumin	TCA protein
Liver	0·82	0	17	1·11 (+35)	37 530	4524
Hepatoma	0·68	0	11	1·13 (+67)	18 252	4935
Cholangioma	0·96	0	15	0·90 (−6)	6 696	4274

Comparison of hepatoma and cholangioma slices

As explained in the Methods section, certain tumours were found by frozen section to consist predominantly of cells of bile-duct origin. The results of incubating cholangioma as well as liver and hepatoma slices with [^{14}C]glycine are shown in Table 4. An increase in serum albumin was found in both liver and hepatoma after 4 hr., but in cholangioma the level of albumin, initially higher than in either of the other tissues, fell slightly. These results, which suggested that little albumin synthesis was taking place in the cholangioma slices, were confirmed by the fact that the radioactivities of albumin in liver and hepatoma were six times and two to three times higher respectively than in cholangioma. On the other hand, the radioactivities of the TCA protein of all three tissues were approximately equal, showing that cholangioma was able to incorporate amino acids into the soluble proteins.

Comparison of liver and kidney slices from normal rats and of liver from tumour-bearing rats

Although there is no evidence that the kidney is able to synthesize albumin *in vivo*, kidney slices contained an appreciable quantity of serum albumin. Kidney therefore seemed to provide a suitable control to test the specificity of the observed synthesis of albumin in liver and tumour tissue. Kidney slices were incubated under the same conditions as had previously been used for liver and tumour. At the same time the opportunity was taken of studying the synthesis of albumin in normal liver slices. Young animals were used for these experiments, since preliminary experiments had shown that the rate of incorporation of radioactive amino acid into liver slices was greater in young animals than in old. As can be seen from Table 5, the results obtained with liver slices from normal animals are closely similar to those from tumour-bearing animals. With kidney slices the level of albumin fell slightly during the incubation and the incorporation of radioactivity into albumin was negligible. On the other hand, there was a considerable incorporation of radioactivity into the soluble-protein fraction, indicating that the tissue was capable of protein synthesis.

Albumin synthesis and secretion in liver and liver-tumour tissue slices

Fig. 7 shows the results of experiments in which the amount of albumin in tumour and liver slices and in the medium was determined during the course of incubation. It will be seen that slices of liver from normal rats and tumour-bearing rats, and slices of liver tumour, all gave similar results in that the total amount of albumin increased, the amount in the slices decreased and the amount in the medium increased. In Table 6 are seen the results of two similar experiments in which the slices were incubated with [^{14}C]glycine, and the incorporation of radioactivity into the albumin in the slices and medium was studied. The specific activity of the albumin in the medium was consistently lower than that in the slices both for liver

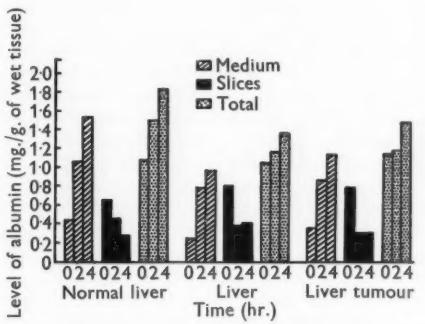


Fig. 7. Levels of albumin (mg./g. of wet tissue) in medium and in slices, and total albumin (medium + slices), during incubation of normal liver slices, slices of liver from tumour-bearing rats and rat-liver tumours. Samples at 0 hr. were stored in an ice-bath for 30 min. before homogenizing the tissue.

Table 5. *Synthesis of albumin and incorporation of radioactivity into albumin and soluble proteins of liver and kidney from normal rats.*

Slices were incubated with 2·5 μc of [^{14}C]glycine. Results are expressed as in Table 4.

	Liver			Kidney		
Time of incubation (hr.)	Amount of albumin (mg.)	Radioactivity		Amount of albumin (mg.)	Radioactivity	
		Albumin	TCA protein		Albumin	TCA protein
0	1·00	378	71	0·99	918	0
2	1·34 (+34)	7 398	1185	0·92 (−7)	756	596
4	1·93 (+93)	12 042	2599	0·88 (−11)	648	839

Table 6. *Incorporation of radioactivity into the serum albumin and soluble proteins precipitated by trichloroacetic acid of medium and slices during incubation of liver and liver-tumour slices with 2·5 μc of [^{14}C]glycine*

Radioactivity is expressed as counts/min./cm.^{2} at infinite thickness. Extracts were not cleared before albumin was precipitated.

Expt. no.	Time of incubation (hr.) ...		Liver 0	Liver 2	Liver 4	Tumour 0	Tumour 2	Tumour 4
V	Albumin	Medium	432	6 264	17 280	216	8 154	16 146
		Slices	108	8 262	19 980	216	12 690	17 064
VI	Albumin	Medium	1458	6 750	16 092	324	4 374	6 210
		Slices	—	15 174	31 536	216	7 452	12 366
	TCA protein	Medium	41	1 260	3 568	38	1 868	3 712
		Slices	—	1 192	1 551	13	2 462	3 071

and tumour, but there was no significant difference between the radioactivity of the TCA protein in the medium and slices.

DISCUSSION

Since the transformation of normal cells into malignant ones involves loss of morphological differentiation there has been a tendency to expect the characteristic biochemical functions of the normal cells to be lost in the process. That this is not invariably so has been shown in a number of tumours of endocrine origin. Thus certain metastases of adenocarcinomas of the thyroid in man retain many of the functions of normal thyroid tissue (Seidlin, Marinelli & Oshry, 1946) and adrenal cortical carcinomas can produce a mineralocorticoid effect (Foye & Feichtmeir, 1955). Hence it seemed possible that tumour cells derived from liver cells might retain the ability to synthesize serum albumin.

Peters & Anfinsen (1950*b*) showed that the amount of serum albumin in the supernatant from homogenates of chicken-liver slices plus medium increased during the course of incubation of the slices in a bicarbonate medium. Furthermore, when the slices were incubated in the presence of radioactive bicarbonate or glycine the albumin became radioactive. They therefore concluded that the slices were synthesizing serum albumin.

The results of the present experiments with rat-liver slices are similar to those of Peters & Anfinsen in that an increase in the amount of albumin in the slices plus medium during the course of incubation was invariably found. This was paralleled by the incorporation of radioactive amino acids into both the mixture of soluble tissue proteins precipitated by trichloroacetic acid and into the serum albumin precipitated by antiserum. The experiments with rat-kidney slices, in which there was a fall in the level of serum albumin during incubation, and a negligible incorporation of radioactive amino acid into the serum albumin, provide good evidence for the specificity of the synthesis observed in liver slices.

Khesin (1952) incubated rat-liver slices under similar conditions to those of Peters & Anfinsen and found that the amount of albumin in aqueous extracts of the slices and medium increased on incubation. However, when he treated homogenates of the slices by a procedure involving butanol he found there was no net increase in albumin in the slices plus medium during incubation. Khesin claims that the butanol procedure releases albumin from the cytoplasmic particles and that the increase in albumin observed with aqueous extracts is due to the release of preformed serum albumin. On the other hand, the levels of serum albumin found by Khesin in aqueous extracts of the slice homogenates were very much lower than those in the present experiments, and he did not study the incorporation of radioactive amino acids.

In the absence of exchange of amino acids with protein-bound amino acids the results of the present experiments must be interpreted as evidence that serum albumin has been synthesized by liver slices. The experiments with liver-tumour slices gave results which were qualitatively very similar to those obtained with liver slices, and it is therefore concluded that tumour slices also synthesize serum albumin. However, it was necessary to determine whether the synthesis in the tumour slices was due to the presence of normal liver cells within the tumour slices. The results of estimations of the amount of normal tissue in the slices on the basis of their content of glycogen, together with histological examination of the slices, suggested that this was not so. The failure of slices consisting mainly of cholangioma cells to synthesize albumin showed that the synthesis was confined to the hepatoma cells within the tumour slices.

Having shown that liver and hepatoma cells in slices behave in a qualitatively similar manner it is now necessary to turn to the quantitative aspects of the results. The result of four typical experiments in which the amount of albumin in liver and tumour

slices was estimated during the course of incubation are shown in Table 1. Although there are variations between the results of the different experiments, certain general conclusions can be drawn. Thus although the percentage increase in albumin in liver and tumour slices was not markedly different, the amount of albumin in tumour slices was consistently less than that in liver slices. For this reason the absolute amount of albumin synthesized in a given time by tumour slices was less than that by liver slices. If the average of the four experiments is taken, then liver slices synthesized 0·72 mg. of albumin/g. of tissue in 2 hr., and 0·94 mg. in 4 hr., whereas the corresponding figures for tumour were 0·26 and 0·48 mg. Thus the tumour slices synthesized between 30 and 50% of the albumin synthesized by a similar weight of liver slices. It is of interest that with slices of chick liver Peters & Anfinsen (1950*b*) found an average increase of 0·12 mg. of chick-serum albumin/g. of tissue/hr., and Campbell (1955) obtained 0·43 mg. in 2 hr. and 0·90 mg. in 4 hr.

In this connexion the results of the determinations of rate of incorporation of radioactive amino acid are of interest. First, however, the effect on the radioactivity of the tissue proteins of incubating different amounts of tissue slices with the same amount of radioactive amino acid must be considered. The degree to which tissue proteins become labelled when slices of tissue are incubated with a radioactive amino acid must depend on the radioactivity of the amino acid present at the site of protein synthesis and the rate of protein synthesis. Thus in order to compare the rate of protein synthesis taking place in slices of different tissues it is necessary to know the relationship between the radioactivity of the amino acid precursor in the various tissues. If the amount of radioactive amino acid added to the medium in which the slices are incubated is large relative to the size of the pools of that amino acid in the slices, then it may be assumed that the radioactivity of the precursor amino acid in the various tissues does not differ widely, and a comparison of the rate of incorporation of that amino acid into the tissue protein will provide a comparison of the rate of synthesis of protein in the tissues. In this case there will be little variation in the degree of radioactivity of the proteins when different weights of tissue are incubated with the same amount of radioactive amino acid. This assumes that the concentration of amino acid is not such as to influence the process of incorporation (see Melchior & Tarver, 1947).

If, on the other hand, the amount of radioactive amino acid added to the medium is small compared with the size of the pools of amino acids in the tissue slices then it cannot be assumed that the radioactivity of the amino acid precursor in the different tissues is the same, and a study of rates of incorporation of radioactivity cannot be interpreted in terms of rates of protein synthesis. In this case the degree of radioactivity of the proteins in the tissue will fall as increasing amounts of tissue are incubated with a constant amount of radioactive amino acid.

The results of experiments designed to determine which of the above conditions applied to the present work are shown in Table 2. In Expt. A it will be seen that the radioactivity of the TCA protein from the liver remained fairly constant, whereas that of the tumour fell, with increasing amounts of tissue. The radioactivity of the albumin from both liver and tumour remained relatively constant in this experiment. In Expt. B, on the other hand, in which similar quantities of [^{14}C]glycine were used, the radioactivity of the TCA protein and albumin from liver and tumour fell with increasing amounts of tissue.

The results of Expt. A suggest that the pool size of glycine in the liver slices is smaller than that in tumour slices, whereas in Expt. B the evidence is that the two tissues are rather similar in this respect since the change in protein radioactivity with amount of tissue is similar in each case. Since the results of the two experiments are not identical it would appear that variations between different batches of slices may be great, and that unless the radioactivity of the precursor amino acid is determined in each case such experiments do not provide a satisfactory method of comparing the rate of protein synthesis in slices of different samples of tissue (for further discussion see Campbell & Halliday, 1957).

In view of the above considerations it is not possible to draw conclusions as to the relative rate of protein synthesis occurring in slices of liver and tumour from the rate of incorporation of radioactive amino acids into the protein of these tissues. However, since the slices from each type of tissue were well mixed before incubation, and the same weight of tissue was placed in each flask in any one experiment, the results obtained at 0, 2 and 4 hr. should be comparable.

When liver and tumour slices were incubated with [^{14}C]glycine the radioactivity of the albumin in the liver was greater than that in the tumour, whereas the reverse was true of the TCA proteins (Table 3). When [^{14}C]glucose replaced glycine the radioactivity of the albumin in the tumour was much greater than that in the liver and the difference between the TCA protein in the two tissues was even greater. Thus when the protein precursor was altered, the pattern of incorporation of radioactivity into the TCA proteins was reflected in a similar manner in the albumins. It therefore seems probable that albumin and the soluble tissue proteins are synthesized from the same pools of radioactive

amino acid. For this reason it is possible to compare the rate of albumin synthesis relative to the synthesis of TCA proteins in the two tissues.

If less albumin is synthesized in the tumour than in the liver slices, as has been suggested, then relative to the radioactivity of the TCA proteins the activity of the albumin should be less. That this is so can be seen from Table 3. After incubation for 4 hr. the TCA protein was 6983 counts in the tumour and 3613 counts in the liver; whereas the albumin in the tumour (11 502 counts) was less active than that in the liver (12 744 counts). If albumin had been synthesized at the same rate as the TCA protein in the two tissues the count of the tumour albumin would have been approximately double, which suggests that albumin was synthesized twice as fast in liver as in tumour. If the absolute amounts of albumin synthesized in the two tissues in this particular experiment are compared (see Table 1, Expt. XI) it will be seen that after 4 hr. the liver slices had synthesized 2·5 times as much albumin as had those from tumour. Thus the radioactivity measurements and the albumin estimates show good correlation, which suggests that the incorporation of radioactive amino acid takes place as a result of the synthesis of complete protein rather than by amino acid/protein-bound amino acid exchange. It may therefore be concluded that if the rate of synthesis of the soluble tissue proteins in liver and liver tumour is similar then serum albumin is synthesized at approximately half the rate in tumour slices that it is in liver slices.

It may be observed that the radioactivity of albumin in the liver slices was invariably much higher than that of the soluble tissue proteins. This is in agreement with the results of Peters & Anfinsen (1950*a*) and Campbell (1955) with chick-liver slices.

It remains to consider the results of the experiments in which the slices were separated from the medium after incubation. Peters (1953) found that when chick-liver slices were incubated with radioactive amino acid, and the radioactivity of the albumin in the medium plus slices was measured during the course of incubation, there was a delay in the incorporation of radioactivity into the albumin, whereas there was no delay in the incorporation of radioactivity into the liver protein as a whole. Peters interpreted these results as indicating that the conversion of amino acids into complete protein requires a measurable period of time and involves a 'polypeptide-like' intermediate.

When rat-liver and tumour slices were incubated with [^{14}C]glycine, the radioactivity of the albumin in the medium was consistently less than that in the slices (see Table 6). On the other hand, there was no such difference between the medium and slices in the activity of the total proteins precipitated by trichloroacetic acid. These results are consistent with those of Peters and show that the activity of the albumin in the medium lags behind that of the mixed tissue proteins. Such a lag could be explained if liver slices release albumin into the medium more rapidly than the other soluble tissue proteins, for relatively more unlabelled albumin would be released. This release of serum albumin seems to be a process requiring energy, for Khesin (1952) has shown that it is slower under anaerobic than under aerobic conditions. Thus albumin would appear to be secreted by the liver and tumour slices.

On the other hand, Peters found that when slices were incubated with radioactive amino acid and were then transferred to a non-radioactive medium the radioactivity of the albumin in the slices plus medium continued to increase, whereas that of the other tissue proteins slowly decreased. Such a result cannot be explained on the basis of a rapid release of albumin but would be expected if amino acids derived from the radioactive tissue proteins were utilized for the synthesis of albumin. The lag in the radioactivity of the albumin in the medium in the present experiments could also be explained on this basis. Further experiments are required to elucidate these points.

SUMMARY

1. The preparation of rat-serum albumin with ammonium sulphate fractionation and zone electrophoresis on columns of 'cellulose' is described. The homogeneity of the albumin was investigated by boundary electrophoresis and immuno-electrophoresis.

2. An antiserum, prepared by injection of rat-serum albumin into rabbits, was used to determine the amount of albumin in extracts of tissue slices which had been incubated in a bicarbonate buffer for periods up to 4 hr.

3. Tissue slices were incubated with [^{14}C]glycine and the incorporation of radioactivity into the soluble tissue proteins, precipitated with trichloroacetic acid, and into the albumin precipitated by antiserum, was studied.

4. A net synthesis of serum albumin occurred during the incubation of slices of liver from normal rats, and of slices of liver and liver tumour from rats bearing liver tumours induced by feeding on 4-dimethylaminoazobenzene. Radioactive amino acids were incorporated into both the soluble tissue proteins and the serum albumin of such slices.

5. Immuno-electrophoretic studies indicate that the albumin present in liver and liver-tumour slices is identical with serum albumin.

6. A net synthesis of serum albumin did not occur when rat-kidney slices were incubated, nor did the serum albumin contained in such slices become radioactive when the slices were incubated

with [^{14}C]glycine. However, the soluble tissue proteins did become radioactive under these conditions.

7. When slices of liver tumours consisting mainly of hepatoma cells were incubated with [^{14}C]glycine the serum albumin in the slices became radioactive and there was a net synthesis of albumin. However, when slices of liver tumours consisting mainly of cholangioma cells were incubated under the same conditions the incorporation of radioactivity into the serum-albumin fraction was very small and there was a net loss of serum albumin during the incubation. Such slices did, however, incorporate radioactivity into the soluble tissue proteins.

8. The incorporation of radioactivity from [^{14}C]glucose by slices of rat liver and liver tumour was also studied.

9. Radioactive rat-serum albumin was prepared from the serum of a rat which had received an injection of [^{14}C]lysine. This albumin was used to determine the combining ratio of serum albumin to antibody in precipitates of these proteins. The ratio was found to vary according to the concentration of albumin in the solution to which the antiserum was added.

10. The effect on the radioactivity of the soluble tissue proteins and serum-albumin fractions of incubating varying amounts of liver and liver-tumour slices with a constant amount of [^{14}C]-glycine was studied.

11. Slices of rat liver and liver tumour were incubated with [^{14}C]glycine and the radioactivity of the albumin and soluble tissue protein fractions was determined in the medium and slices independently. The results are interpreted as suggesting that serum albumin is 'secreted' both by liver slices and by liver-tumour slices.

12. From a comparison of the results obtained with liver slices and liver-tumour slices it is concluded that the hepatoma cells in liver tumours retain the ability of the liver cells to synthesize serum albumin. Determinations of the absolute amount of serum albumin synthesized by slices indicate that the tumour slices synthesize only 30–50 % as much albumin as do a similar weight of liver slices. Studies on the rate of incorporation of radioactive amino acid into the serum albumin in the tissues are in agreement with this observation.

We should like to thank Professor F. Dickens, F.R.S., for his interest and advice, Dr A. C. Thackray of the Bland-Sutton Institute of Pathology for advice concerning the histology of the tumours, and the British Empire Cancer Campaign for a grant to the Medical School. One of us (P.N.C.) is also grateful to Professor G. Ågren of the Institute of Medical Chemistry at Uppsala for the opportunity to meet Dr J. Porath, who gave much valuable advice concerning the use of his cellulose columns for preparative electrophoresis. Dr R. A. Kekwick and Miss M. E. Mackay, of the Lister Institute, offered advice on the preparation of rat-serum albumin. Dr G. Bennette, Dr I. M. Roitt and Mr H. E. H. Jones also assisted in various aspects of the work. Mr M. Woodford prepared the 3′-methyl-*p*-dimethylaminoazobenzene and Miss A. Doughty carried out the electrophoresis analyses in the Tiselius apparatus. To all these friends we would like to express our thanks. We are also grateful to Miss B. Kernot for her skilled assistance throughout the course of the work.

REFERENCES

Askonas, B. A., Campbell, P. N., Humphrey, J. H. & Work, T. S. (1954). *Biochem. J.* **56**, 597.
Campbell, P. N. (1955). *Biochem. J.* **61**, 496.
Campbell, P. N. & Halliday, J. W. (1957). *Biochem. J.* **65**, 28.
Campbell, P. N. & Stone, N. E. (1955). *Biochem. J.* **61**, xx.
Campbell, P. N. & Stone, N. E. (1956). *Biochem. J.* **62**, 9P.
Cohn, E. J., Strong, L. E., Hughes, W. L. jun., Mulford, D. J., Ashworth, J. N., Melin, M. & Taylor, H. L. (1946). *J. Amer. chem. Soc.* **68**, 459.
Edwards, J. E. & White, J. (1941). *J. nat. Cancer Inst.* **2**, 157.
Fairbairn, N. J. (1953). *Chem. & Ind.* p. 86.
Flodin, P. & Porath, J. (1954). *Biochim. biophys. Acta*, **13**, 175.
Foye, L. V. jun. & Feichtmeir, T. V. (1955). *Amer. J. Med.* **19**, 966.
Grabar, P. & Williams, C. A. jun. (1955). *Biochim. biophys. Acta*, **17**, 67.
Hagedorn, J. C. & Jensen, B. N. (1923*a*). *Biochem. Z.* **135**, 46.
Hagedorn, J. C. & Jensen, B. N. (1923*b*). *Biochem. Z.* **137**, 92.
Hoch-Ligeti, C., Hoch, H. & Goodall, K. (1949). *Brit. J. Cancer*, **3**, 140.
Kahan, J. (1953). *Arch. Biochem. Biophys.* **47**, 408.
Keltz, A. & Mehl, J. W. (1954). *J. Amer. chem. Soc.* **76**, 4004.
Khesin, R. V. (1952). *C.R. Acad. Sci. U.R.S.S.* **84**, 1209.
Melchior, J. & Tarver, H. (1947). *Arch. Biochem.* **12**, 309.
Miller, L. L., Bly, C. G., Watson, M. L. & Bale, W. F. (1951). *J. exp. Med.* **94**, 431.
Oudin, J. (1948). *Ann. Inst. Pasteur*, **75**, 30, 109.
Peters, T. jun. (1953). *J. biol. Chem.* **200**, 461.
Peters, T. jun. & Anfinsen, C. B. (1950*a*). *J. biol. Chem.* **182**, 171.
Peters, T. jun. & Anfinsen, C. B. (1950*b*). *J. biol. Chem.* **186**, 805.
Porath, J. (1954). *Acta chem. scand.* **8**, 1813.
Price, J. M., Harman, J. W., Miller, E. C. & Miller, J. A. (1952). *Cancer Res.* **12**, 192.
Seidlin, S. M., Marinelli, L. D. & Oshry, E. (1946). *J. Amer. med. Ass.* **132**, 838.
Ulrich, F., Li, C. H. & Tarver, H. (1954). *Arch. Biochem. Biophys.* **50**, 421.
Zamecnik, P. C. (1952). *Annu. Rev. Biochem.* **21**, 411.
Zamecnik, P. C., Loftfield, R. B., Stephenson, M. L. & Steele, J. M. (1951). *Cancer Res.* **11**, 592.

Studies of Sebum

8. OBSERVATIONS ON THE SQUALENE AND CHOLESTEROL CONTENT AND THE POSSIBLE FUNCTIONS OF SQUALENE IN HUMAN SEBUM*

By BARBARA BOUGHTON, R. M. B. MacKENNA, V. R. WHEATLEY and A. WORMALL
Departments of Biochemistry and Dermatology, Medical College of St Bartholomew's Hospital, London, E.C. 1

(*Received* 16 *October* 1956)

The object of our investigations has been to study the composition of sebum and to elucidate its role in maintaining the health of the skin. In a previous paper on the hydrocarbons of sebum (Boughton, Hodgson-Jones, MacKenna, Wheatley & Wormall, 1955) we observed that the squalene content of sebum showed wide variations, both in samples collected from the same subject on different occasions and between samples collected from different subjects. This variation may be peculiar to squalene or it may indicate a variation in the overall composition of sebum. To investigate this possibility, we have, therefore, studied the comparable variations in another constituent of sebum, cholesterol, and have also extended our observations on squalene in a study of the possible role of this hydrocarbon in the metabolism of the skin. A preliminary report of part of this work has already appeared (MacKenna, Wheatley & Wormall, 1955).

EXPERIMENTAL

Estimation of the squalene and cholesterol contents of sebum. Samples of sebum were collected by the polythene-cup method already described (Boughton *et al.* 1955). The cup was made from the upper part of a polythene filter funnel 6 cm. in diameter, cut across about 2·5 cm. from the top. The cup was pressed against the body and the area of skin enclosed extracted with one or two portions of carbon tetrachloride (glass-distilled). Several adjacent areas of skin were extracted in this manner in order to obtain samples sufficiently large for analysis, and the combined extracts were filtered, evaporated to small bulk under reduced pressure and then diluted to 15 ml. with carbon tetrachloride. Suitable samples of this solution were used for analysis. The sebum content of the solution was estimated by evaporating a sample in a tared microflask and weighing the residue (Hodgson-Jones & Wheatley, 1952), squalene was estimated by the iodometric method (Wheatley, 1953) and cholesterol by the Liebermann–Burchardt method. It was observed, however, that the iodometric titration of squalene was unreliable with amounts of squalene of less than 75 μg.; if therefore the thiosulphate titration required less than 0·5 ml. of 0·005 N thiosulphate the determination was repeated with a larger sample.

* Part 7: Wheatley & James (1957).

Combination with SH *groups and inhibition of enzymes.* The colorimetric method (Kun & Abood, 1949) was found to be unsuitable for the estimation of succinic dehydrogenase in the presence of squalene, owing to the preferential solubility of the coloured complex in the squalene. Manometric methods were therefore used for the determination of succinic dehydrogenase, cytochrome oxidase, urease, and of whole-yeast fermentation. The SH groups of glutathione solutions were estimated by the ferricyanide method of Mason (1930).

Depilatory studies. The depilatory effect of a number of substances was studied. The test substance (1 ml.) was pipetted on to the skin of a rabbit and lightly rubbed in with the tip of the finger. The site chosen for most experiments was the top of the head between the ears since this site is the least accessible to the animal. It is possible, however, that the hair of this region may be slightly different from that of the rest of the body, and, in order to exclude this possibility, experiments were performed in which the same test substance was applied simultaneously to the head and the centre of the back. After application of the test substance the site of application was examined at daily intervals until hair loss occurred. Preliminary experiments were also performed with the rat, mouse or guinea pig as the test animal, but these animals proved to be much less sensitive to the substances producing depilation than did the rabbit.

Fungicidal and bactericidal activity of squalene. Tests for fungicidal and bactericidal activity were performed for us on squalene from three different sources by Professor L. P. Garrod in the following manner. (1) Glass beads on which *Staphylococcus aureus* or *Candida albicans* had been dried were immersed in squalene and individual beads were removed and cultivated at intervals up to 24 hr. (2) Mixtures of squalene and broth (10–0·07% squalene) with Tween 80 (polyoxyethylene sorbitan mono-oleate) to ensure admixture were incubated for 24 hr. and then plated. (3) Plates of Sabouraud's medium were poured with a thin top layer pre-inoculated with *C. albicans* or *Aspergillus niger*. Before incubation, undiluted squalene was applied to these either in cylinders or simply by allowing a drop to fall on the surface of the medium.

RESULTS

Squalene content of sebum

Normal values. Estimations were made on the sebum from the backs of normal subjects (20 children aged 1–12 years, 20 adult females aged 13–38 and 20 adult males aged 18–74) and the

results are summarized in Table 1. These results show that the squalene content of sebum is significantly lower in children than in adults ($P < 0.001$) but there is no significant difference between the values for adult males and those for adult females ($P > 0.05$). Wide variations in normal subjects were found to occur and this was much more marked in the children than in adults. A dot diagram was plotted of the squalene content and age but no change was found to occur at any age group.

Daily variations. Determinations were made on the sebum collected from the backs of normal subjects, five females and three males. The samples were collected at daily intervals (excluding weekends) for periods varying from 14 to 28 days. The typical results of a male and a female subject (Fig. 1) indicate the appreciable variation encountered. The range of squalene content from the other six subjects was as follows: the four females, 10·6–15·2, 10·0–12·4, 8·8–11·6 and 6·8–8·8 respectively, and the two males 4·9–10·2 and 6·3–9·5. In the female subjects the changes in squalene content could not be related to the menstrual cycle (see Fig. 1).

Variations over the surface of the body. Samples were collected from a group of normal subjects, four males and four females, from five different areas of the body of each subject, namely, the back, chest, abdomen, arms and legs (Table 2); all the samples from a particular subject were collected on one day and as far as possible during the same part of the day. In view of the wide variations already encountered, detailed analysis of these results has not been attempted, but in practically every case the squalene content of the sebum from the back and chest was consistently higher than that from other areas.

Correlation with sebum level. A dot diagram (Fig. 2) of the squalene content of sebum plotted against the sebum level (μg. of sebum covering 1 cm.² of skin) showed that for a sebum level greater than 40 μg./cm.² the squalene content was independent of the sebum level. For sebum levels below this limit the squalene content dropped as the sebum level fell.

Oxidation of squalene in thin films in vitro *and* in vivo. One explanation of the wide variations in the squalene content of sebum could be that squalene is oxidized after secretion on to the skin surface and that the variations are due to different rates of oxidation caused by variations in local conditions. To test this hypothesis, thin films were prepared by evaporating squalene-containing fatty materials dissolved in carbon tetrachloride in a stream of nitrogen in 100 ml. flasks, kept constantly rotated during the evaporation in order to spread the lipid over the walls of the flasks. These were then set aside and the lipid was allowed to oxidize at room

Table 1. *Squalene content of the sebum from the backs of normal subjects*

Subjects	No. of subjects	Squalene content of sebum (%) Range	Average (and S.D.)
Children	20	1·7–13·8	6·3±3·3
Adults, male	20	6·5–13·4	9·3±2·1
Adults, female	20	7·0–13·0	10·2±1·4

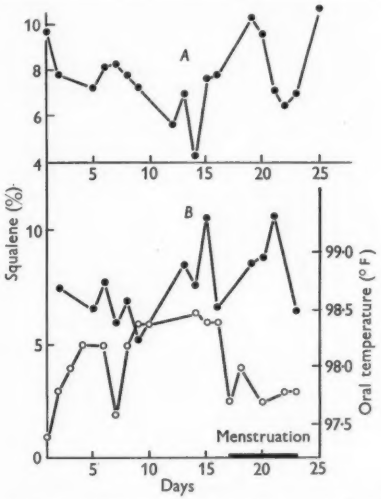


Fig. 1. Daily variation in the squalene content of the sebum from the backs of two normal subjects. Curve *A*, male subject. Curve *B*, female subject. ●, Squalene content; the basal-temperature curve (○) has been included to confirm the normal nature of the menstrual cycle.

temperature for fixed periods, at the end of which a squalene estimation was performed. Experiments made with pure squalene, fresh sebum, and sebum to which squalene had been added showed that under these conditions only a negligible amount of squalene was oxidized in 24 hr.

To test the possibility of squalene being oxidized on the skin surface the following experiments were made. A sample of sebum was collected from the back of the subject in the usual manner. The back was then defatted completely by means of a suitable solvent, and after allowing a convenient interval of time to elapse (2–4 hr.) for sufficient sebum for analysis to be secreted, a second sample was collected. The squalene contents of both samples were determined. The second sample had remained on the skin surface for only a limited time, and its squalene content would be expected to be appreci-

Table 2. *Squalene content of the sebum from various areas of the body*

Subject	Sex	Squalene content of sebum (%) Back	Chest	Abdomen	Arm	Leg
J.C.C.	M.	10·3	9·9	5·4	6·8	6·4
J.D.H.	M.	8·6	10·2	5·2	9·1	11·5
R.T.	M.	11·1	11·3	8·3	10·4	5·9
V.R.W.	M.	11·0	11·5	7·5	5·8	6·7
J.H.	F.	9·0	8·0	6·5	6·0	6·1
E.B.	F.	9·9	10·8	7·3	6·0	7·9
B.B.	F.	8·9	12·0	5·2	6·2	3·9
P.H.S.	F.	9·8	9·6	6·4	6·6	9·1
Average		9·8	10·4	6·5	7·1	7·2
Standard deviation		0·9	1·3	1·2	1·7	2·0

Table 3. *Squalene content of sebum samples collected at short intervals after defatting of the skin compared with the squalene content of casually collected samples*

Subject	Solvent used to 'defat' skin	Squalene content (%) Before defatting	After defatting (*a*)*	(*b*)*	(*c*)*
M.E.L.	CCl_4	7·7	7·5	—	—
B.B.	CCl_4	8·5	8·3	—	—
E.B.	Acetone	10·4	—	12·9	—
A.R.	Acetone	12·3	—	12·7	—
M.E.L.	Acetone	6·8	—	—	8·3
M.E.L.	Light petroleum	7·2	—	—	9·2
B.B.	Light petroleum	11·5	—	—	13·7

* (*a*), (*b*) and (*c*) were collected after intervals of 2, 3 and 4 hr. respectively.

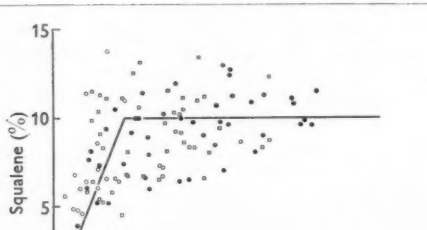


Fig. 2. Relationship between squalene content of sebum and the sebum level of the area of skin from which the sample was collected of adult male (□) and female (●) subjects, and of children (○).

ably higher than that of the casually collected sample if the squalene in sebum on the skin surface is extensively oxidized. The results (Table 3) showed that the squalene content of the second sample was usually slightly higher than that of the first sample, but the difference was often negligible. The most reasonable interpretation is that while some oxidation of squalene may take place on the skin surface it is not sufficient to explain the wide variations in the squalene content of sebum.

Inhibition of enzymes by squalene. Flesch (1951) observed an inhibition of succinic dehydrogenase by squalene, which he attributed to a combination with the SH groups of the enzyme. Dr E. D. Wills kindly undertook to investigate this inhibition for us. The effect of squalene on succinic dehydrogenase, urease, cytochrome oxidase (a non-SH enzyme) and whole-yeast respiration were studied manometrically. The results (Table 4) show that there is no inhibition of succinic dehydrogenase and only slight inhibition of urease by relatively large quantities of squalene. Slight inhibition of whole-yeast respiration occurred but this may well have been due to a surface effect.

Combination with SH groups. The possible combination of SH groups with squalene was studied for us by Miss P. Handoll. Estimations were performed on solutions of glutathione in the presence and absence of squalene with sodium dodecyl sulphate as an emulsifying agent. No evidence of combination with the SH of glutathione was observed.

Depilatory action of squalene and other lipids. The depilatory action of a variety of lipids and other substances was tested on the rabbit with the results shown in Table 5. Many of these fatty materials had a depilatory effect similar to that of squalene and this activity was not confined to the unsaturated compounds only. The time required for the hair loss to be noticeable after the application of the test substance varied appreciably in these experiments,

Table 4. *Possible inhibition of certain enzymes by squalene*

Enzyme system	Squalene (ml.)*	Oxidized squalene† (ml.)*	Inhibition (%)
Succinic dehydrogenase from pig heart	0·5	—	0
	0·25	—	0
	0·1	—	0
	—	0·5	0
	—	0·2	0
	—	0·1	0
Succinic dehydrogenase from rat liver	0·5	—	0
	0·25	—	0
	0·1	—	0
	—	0·5	0
Urease (cryst.)	1·0	—	15
	0·75	—	15
	0·5	—	15
	—	1·0	15
Cytochrome oxidase	1·0	—	0
	0·5	—	0
	0·25	—	0
	0·1	—	0
Whole-yeast fermentation	1·0	—	23
	0·5	—	8

* Amount of squalene or oxidized squalene added to each manometer, in which the total aqueous phase was 3 ml.
† Squalene which had been allowed to autoxidize for several months.

Table 5. *Depilatory action on the rabbit of squalene and some other lipids*

Substance	No. of animals: Tested	No. of animals: Showing hair loss	Depilatory effect*
Squalene	8	7	+ +
Liquid paraffin B.P.	5	1	±
Hair dressing†	4	3	+
Castor oil	2	1‡	+
Oleic acid	5	5	+ + +
Methyl oleate	3	3	+ + +
Methyl stearate	3	3	+ +
Olive oil	4	2	+
Shark-liver oil	3	3	+ +
Benzene	3	3	+ +

* Assessed from the appearance of the treated area after hair loss.
† A proprietary brand containing liquid paraffin.
‡ One doubtful.

Table 6. *Cholesterol content of the sebum from the backs of normal subjects*

Subjects	No. of subjects	Cholesterol content of sebum (%): Range	Cholesterol content of sebum (%): Average (and S.D.)
Children	20	5·8–36·3	16·0±5·3
Adults, male	20	2·1–5·4	3·1±0·7
Adults, female	20	2·3–5·2	3·1±0·6

but it was usually about 7 days with most of the substances; with olive oil, the hair dressing, benzene and occasionally with squalene, the period was longer (10–15 days). In the experiments in which the test substance was applied simultaneously to the top of the head and the centre of the back no difference in the depilatory effect on the two areas was observed.

Bactericidal and fungicidal action. No inhibition of the growth of the bacterium *Staph. aureus* or of the fungi *C. albicans* or *A. niger* by squalene was observed under the conditions studied.

Cholesterol content of sebum

Normal values. Estimations made on sebum from the backs of normal subjects (20 children aged 1–12 years, 20 female adults aged 19–48 and 20 male adults aged 19–72) showed (Table 6) that the cholesterol content of children's sebum is markedly higher than that of adults. The results for adult males and females were almost identical and there was no general correlation between cholesterol content and the age of the subject. One subject, aged 75, who had obvious senile changes of the skin (seborrhoeic warts, etc.) gave a very high value (32·7 %) which was not included in the present series.

Daily variations. Daily determinations were made on three male and three female subjects. With one exception all showed little daily variation and a typical result is shown in curve *A* (Fig. 3). The subject exhibiting wide daily variation (curve *B*, Fig. 3) had a consistently low sebum level on the back (11–27 μg./cm.^{2}).

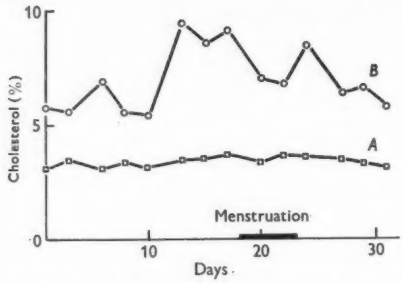


Fig. 3. Daily variations in cholesterol content of the sebum from the backs of two normal adults. Curve *A*, results with a male; *B*, those with a female subject. Two other males and two females gave curves similar to *A*.

Variations over the surface of the body. The cholesterol content of samples of sebum collected from the back, chest, abdomen, arms and legs of eight subjects, four male and four female, are given in Table 7. Here again the subject (B.B.) who exhibited wide daily variations in cholesterol content also gave consistently high values for all areas, whereas in the other subjects the values for the back and chest were generally lower than for other areas.

Correlation with sebum level. A dot diagram of the cholesterol content of sebum plotted against the sebum level (Fig. 4) showed a striking correlation. For high sebum levels the cholesterol concentration in the sebum was very constant and was independent of the sebum level, but with low sebum levels the cholesterol content increased rapidly as

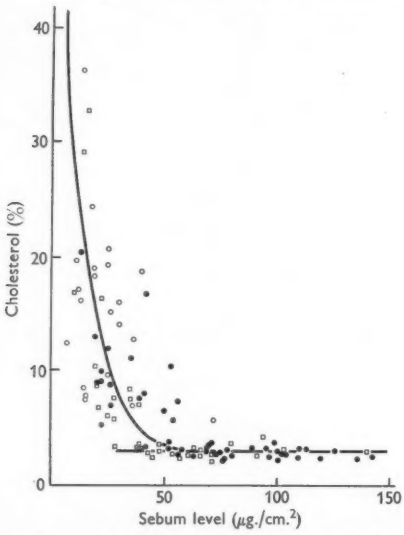


Fig. 4. Relationship between cholesterol content of sebum and the sebum level of the area of skin from which the sample was collected. Adult male subjects (□), adult females (●) and children (○).

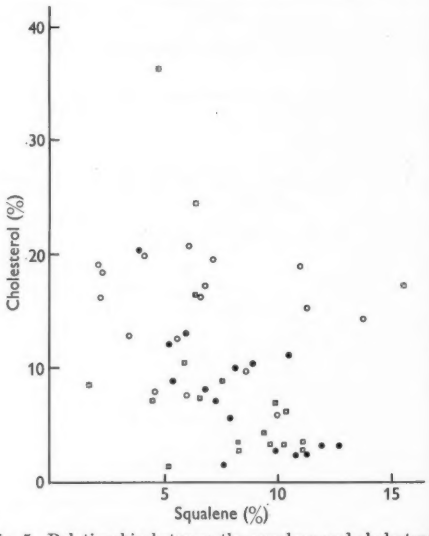


Fig. 5. Relationship between the squalene and cholesterol contents of sebum samples collected from adult male (□) and female (●) subjects, and children (○).

Table 7. *Cholesterol content of the sebum from various areas of the body*

Subject	Sex	Cholesterol content of sebum (%) Back	Chest	Abdomen	Arm	Leg
J.C.C.	M.	3·3	3·8	8·8	8·8	16·3
J.D.H.	M.	2·8	2·4	8·8	5·8	16·9
R.T.	M.	3·5	3·3	3·5	6·1	10·4
V.R.W.	M.	3·3	4·2	7·3	7·1	8·3
J.H.	F.	3·1	3·3	3·9	3·7	10·0
E.B.	F.	2·7	2·5	7·1	13·0	5·7
B.B.	F.	10·3	16·8	12·0	12·6	20·4
P.H.S.	F.	2·4	2·6	6·5	7·6	11·1
Average		3·9	4·9	7·2	8·0	12·4
Standard deviation		2·6	4·9	2·7	3·2	5·0

the sebum level fell. The limiting value dividing the two types of results was about 50 μg./cm.^{2}. The sebaceous activity of the subject, of which the sebum level gives a rough indication, thus markedly influences the cholesterol content of the film of sebum on the skin surface.

Squalene–cholesterol correlation. A dot diagram (Fig. 5) showed that although there was a general tendency for the cholesterol content to increase as the squalene content fell there was no convincing relationship between these two components of sebum.

DISCUSSION

In a previous paper (Boughton *et al.* 1955) we summarized the known and suggested biochemical actions and functions of squalene. These are (*a*) its role as an intermediary in sterol metabolism (Langdon & Bloch, 1953; Cornforth & Popják, 1954), (*b*) its ability to inactivate free SH groups and to inhibit SH-containing enzymes (Flesch, 1951), (*c*) its action as a reversible depilatory when applied to certain animals (Flesch, 1951) and (*d*) its fungistatic action against certain dermatophytes (Sobel, Marmorston & Arzangoolian, 1954). In the present study an attempt has been made to investigate certain of these reported actions of squalene. We have been unable to confirm the claims that squalene is able to inactive SH groups and inhibit SH-containing enzymes, and that it has a significant bactericidal or fungicidal action. When applied to the skin of rabbits it caused reversible hair loss, but similar hair loss was also caused by a variety of saturated and unsaturated lipids and similar substances. We were unable to demonstrate any convincing hair loss when squalene was applied to guinea pig, mouse or rat skin. These results are in agreement with those of Kandutsch & Baumann (1955) who failed to cause depilation of the mouse by repeated application of undiluted squalene, though they did observe some hyperplasia of the epidermis. In previous work (Boughton *et al.* 1955) we did not observe any action on pigmentation or hair growth when squalene was applied to the skin of normal human subjects, nor did it show any therapeutic action when used to treat, by topical application, certain diseases of the skin. We have, therefore, been unable to assign a direct action on the skin which is peculiar to squalene. It has, however, been shown recently by El Ridi, Azouz & Hay (1955) that squalene is essential for promoting lactation in the rat and, since the mammary gland can be regarded as a modified sebaceous gland, an indirect action of squalene on sebaceous activity is still possible.

The wide variations in the squalene content of sebum, both from day to day and between different individuals, have been confirmed. There is, however, no evidence for wide overall variations in sebum composition, since the cholesterol content of sebum appears to be remarkably constant. No explanation for this variation in the squalene content of sebum has been found; from the evidence available it appears probable that the variations are not due solely to variation in the rates of oxidation of squalene once it reaches the surface of the skin. The simple excretion through the skin of an excess of squalene over that required in sterol synthesis cannot be excluded.

The cholesterol in the film of lipid on the skin surface arises from two sources, the sebaceous glands and the cells of the keratinizing epidermis. In other work (Hodgson-Jones, MacKenna & Wheatley, 1952) little evidence was obtained that the amount of lipid arising from the horny-layer cells of the epidermis was significant in comparison with that coming from the sebaceous glands, and we have therefore referred to this lipid film as sebum. We have now shown that for low sebum levels the amount of cholesterol arising from the epidermis can appreciably alter the composition of the surface film. We can, therefore, no longer regard the surface skin lipid collected from areas of skin with low sebum levels, e.g. the arms and legs, as being pure sebum. For experimental work in which the composition of the sebum of different subjects is being compared, sites of high sebaceous activity, e.g. the back, should be used. Even then measurement of the 'sebum level' of the area should also be made and due consideration given to the possible contamination with epidermal lipid should the sebum level fall below the critical value of 50 μg./cm.^{2}. This finding also explains why the cholesterol content of the 'sebum' of children is higher than that of the adult, since the 'sebum' of children, who generally have 'sebum levels' below the critical limit, is appreciably diluted with epidermal cholesterol. A similar explanation has recently been put forward by Nicolaides & Rothman (1955).

Study of the variations of only two components of sebum has proved to be of limited value. Histochemical and other evidence indicates that appreciable lipid synthesis takes place in the sebaceous gland; in order to be able to detect changes in this metabolic activity due to disease and other factors a much more detailed study of the overall variations in sebum composition is required. Until recently such a study has not been possible owing to a lack of suitable small-scale methods of lipid analysis; recent advances (James & Martin, 1956; James & Wheatley, 1956), however, have made such analysis possible, and a much more detailed investigation of the variations in sebum composition is now being undertaken.

SUMMARY

1. The squalene and cholesterol contents of the sebum from the backs of normal children and adults have been studied. It has been confirmed that the squalene content of sebum is lower and the cholesterol content higher in children than in adults.

2. A relationship between the sebaceous activity of a particular area of skin and the squalene and cholesterol content of the sebum derived therefrom has been demonstrated. It is considered that dilution of the surface-film lipids by lipids from the horny-layer cells significantly alters the composition of the 'sebum' in areas of skin with low sebaceous activity, e.g. most skin areas of the child and the arms and legs of adults. This apparent dilution could account for the observed differences between the squalene content of the 'sebum' from children and that of adults. To avoid this dilution effect, it is suggested that for studies of the composition of sebum the sample should be collected from the back.

3. The wide variations in the squalene content of sebum, both from day to day and between different individuals, has been confirmed. This is not due to epidermal contamination of the sample nor solely to variable rates of oxidation of squalene in different samples of sebum.

4. Claims that squalene can inactivate SH groups and inhibit certain enzymes have not been confirmed, and squalene does not appear to possess significant fungicidal or bactericidal properties. It can cause reversible depilation in the rabbit but not in the guinea pig, mouse or rat, and this property is not specific for squalene but is shared by a number of saturated and unsaturated lipids.

The authors wish to express their thanks to the many students and staff of this College who have acted as subjects for these investigations, and to Dr H. J. Channon of Unilever Ltd., who provided us with a generous supply of squalene. We are indebted to the Treasurer and Board of Governors of St Bartholomew's Hospital for a grant from the Hospital Discretionary Fund. We are also indebted to Professor L. P. Garrod for collaboration in the experiments involving tests of the bactericidal and fungicidal power of squalene and to Dr E. D. Wills for help with the enzyme tests.

REFERENCES

Boughton, B., Hodgson-Jones, I. S., MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1955). *J. invest. Derm.* **24**, 179.

Cornforth, J. W. & Popják, G. (1954). *Biochem. J.* **58**, 403.

El Ridi, M. S., Azouz, W. M. & Hay, A. A. (1955). *Hoppe-Seyl. Z.* **299**, 283.

Flesch, P. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 801.

Hodgson-Jones, I. S., MacKenna, R. M. B. & Wheatley, V. R. (1952). *Acta derm.-venereol., Stockh.*, **32** (suppl. 29), 155.

Hodgson-Jones, I. S. & Wheatley, V. R. (1952). *Biochem. J.* **52**, 460.

James, A. T. & Martin, A. J. P. (1956). *Biochem. J.* **63**, 144.

James, A. T. & Wheatley, V. R. (1956). *Biochem. J.* **63**, 269.

Kandutsch, A. A. & Baumann, C. A. (1955). *Cancer Res.* **15**, 128.

Kun, E. & Abood, L. G. (1949). *Science*, **109**, 144.

Langdon, R. G. & Bloch, K. (1953). *J. biol. Chem.* **200**, 135.

MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1955). *J. Physiol.* **127**, 36P.

Mason, H. L. (1930). *J. biol. Chem.* **86**, 623.

Nicolaides, N. & Rothman, S. (1955). *J. invest. Derm.* **24**, 125.

Sobel, H., Marmorston, J. & Arzangoolian, H. (1954). *Science*, **119**, 816.

Wheatley, V. R. (1953). *Biochem. J.* **55**, 637.

Wheatley, V. R. & James, A. T. (1957). *Biochem. J.* **65**, 36.

Metabolism of Polycyclic Compounds

11. THE CONVERSION OF NAPHTHALENE INTO 2-HYDROXY-1-NAPHTHYL SULPHATE IN THE RABBIT*

BY E. BOYLAND AND P. SIMS

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W. 3

(*Received* 18 *October* 1956)

By the use of paper-chromatographic analysis, Corner & Young (1954, 1955), Sato, Yamada, Suzuki, Fukuyama & Yoshikawa (1956) and Solomon (1955) detected the presence of a metabolite of naphthalene in the urine of rats dosed with naphthalene, which gave the colour reactions of 1:2-dihydroxynaphthalene after the chromatograms had been treated with mineral acid. By injecting ^{35}S-labelled sodium sulphate into the dosed animals, the first two groups of workers were able to show by the use of radiochromatograms that the metabolite was probably a sulphuric ester of 1:2-dihydroxynaphthalene. Furthermore, Corner & Young (1955) detected 1:2-dihydroxynaphthalene

* Part 10: Boyland & Solomon (1956).

after the urines had been heated with acid, and Sato *et al.* (1956) found that the incubation of 1:2-dihydroxynaphthalene and ^{35}S-labelled sulphate with liver slices also yielded the new metabolite. All the above workers found that the metabolite was also produced when rats were treated with *trans*-1:2-dihydronaphthalene-1:2-diol. Corner & Young (1954) showed that the new metabolite, which must be either 2-hydroxy-1-naphthyl sulphate or 1-hydroxy-2-naphthyl sulphate, was also present in the urine of guinea pigs, mice and rabbits dosed with naphthalene and they detected free 1:2-dihydroxynaphthalene in the guinea-pig urine.

The present work describes the isolation of 2-hydroxy-1-naphthyl sulphate from the urine of rabbits dosed with naphthalene, which was identical with the synthetic ester prepared by the Elbs persulphate oxidation of 2-naphthol. Desai & Sethna (1951) isolated 1:2-dihydroxynaphthalene from this oxidation.

EXPERIMENTAL

1:2-*Dihydroxynaphthalene.* This was prepared by the reduction of 1:2-naphthaquinone with sodium dithionite by the method of Fieser & Fieser (1939). The crude product was purified by distillation in a microsublimation apparatus (180° at 12 mm.), to yield 1:2-dihydroxynaphthalene in yellowish needles, m.p. 102°. The diacetate separated from ethanol in prisms, m.p. 109° (Found: C, 68·8; H, 5·2. Calc. for $C_{14}H_{12}O_4$: C, 68·8; H, 4·95%). Fieser & Peters (1931) recorded m.p. 109·5° for this compound.

Potassium and sodium 2-hydroxy-1-naphthyl sulphate. 2-Naphthol (10 g.) and KOH (10 g.) in water (100 ml.) were cooled to 0° and a saturated aqueous solution of $K_2S_2O_8$ (20 g.) was added during 8 hr. with continuous stirring. The mixture was kept overnight at room temperature, the dark solid was filtered off and discarded, and the filtrate was adjusted to pH 4 with acetic acid. The solution was washed with ether (3 × 100 ml.) to remove unchanged 2-naphthol, and passed through a bed of charcoal (100 g., activated, British Drug Houses Ltd.) supported in a glass chromatography tube. The bed was washed with water (2 l.) to remove inorganic sulphate, with aqueous 2N-NH_3 soln. (1 l.), which removed coloured materials, and finally with methanol (1·5 l.) to elute the sulphuric ester. The methanolic eluate was evaporated to dryness under reduced pressure and the residue was recrystallized from aqueous ethanol to yield *potassium 2-hydroxy-1-naphthyl sulphate* (3·4 g.) in elongated plates (Found: C, 43·2; H, 2·8; S, 10·9; K, 13·9. $C_{10}H_7O_5SK$ requires C, 43·15; H, 2·5; S, 11·5; K, 14·05%).

When the potassium salt (1 g.) in 5N-HCl (5 ml.) was heated to 100° for 30 min. and cooled to 0°, 1:2-dihydroxynaphthalene (380 mg.) separated in glistening plates (m.p. 58–59°), which darkened on exposure to air. The crystals were dried at 100° at 0·2 mm. and recrystallized from light petroleum (b.p. 60–80°) in needles, m.p. and mixed m.p. 98–100°. The diacetate formed prisms from ethanol, m.p. and mixed m.p. 109°. The mother liquors from the hydrolysis were shown, by the use of $BaCl_2$, to contain inorganic sulphate.

The potassium salt (2 g.) was dissolved in 5N-NaOH (10 ml.) and ethanol (50 ml.) was added. The solution was warmed for a few minutes and cooled to 0°, when a substance which appeared to be the disodium salt of 2-hydroxy-1-naphthyl sulphate separated, which formed silky needles from aqueous ethanol. This substance was hygroscopic and proved unsuitable for analysis. A concentrated aqueous solution of the salt was therefore acidified to pH 2 with conc. HCl, when *sodium 2-hydroxy-1-naphthyl sulphate* separated. From methanol–ether it formed plates (Found: C, 45·6; H, 2·9; S, 11·7; Na, 9·0. $C_{10}H_7O_5SNa$ requires C, 45·8; H, 2·7; S, 12·2; Na, 8·8%). The sodium salt was hydrolysed with 5N-HCl and the product was purified as before to yield 1:2-dihydroxynaphthalene, m.p. and mixed m.p. 100–101°.

Properties of 2-hydroxy-1-naphthyl sulphate on paper chromatograms. On descending chromatograms on Whatman no. 1 chromatography papers, the ester had R_F 0·48 when the papers were developed with *n*-butanol saturated with 2N-NH_3 soln., R_F 0·45 with the organic phase of a mixture of *n*-butanol–ethanol–water (17:3:20, by vol.) (Solomon, 1955), and R_F 0·36 with the organic phase of the mixture *iso*butanol–3N-$(NH_4)_2CO_3$–3N-NH_3 soln. (4:3:3, by vol.) (Corner & Young, 1954). The ester appeared as a bright-violet fluorescent spot when the papers were examined under a Hanovia Chromatolite ultraviolet lamp fitted with a Chance OX7 filter. When the chromatograms were sprayed with diazotized *p*-nitroaniline (0·2% in 0·1N-HCl), diazotized sulphanilic acid (0·05% in 0·1N-HCl) or 2-chloro-4-nitrobenzenediazoniumnaphthalene-2-sulphonate (N.N.C.D. reagent; a saturated solution in 0·1N-HCl), followed in each case by aqueous 10% Na_2CO_3, the ester developed little or no colour within 4 hr., but later pale-brown colours appeared. When the papers were sprayed with 2N-HCl and heated in an oven to 70° for 20 min., the ester spot gave immediate purple colours (characteristic of free 1:2-dihydroxynaphthalene) with each of the reagents.

Dosing of animals and collection of urine. Four rabbits (body weight approximately 2 kg.), maintained on oats, rat cake, bread and water, were each injected intraperitoneally on four consecutive days with a solution of naphthalene in arachis oil (5 ml. of 20%, w/v). The animals were housed in metabolism cages designed to allow the collection of urine separately from faeces. The urines were collected up to the sixth day and were stored at 0°.

Isolation experiments. The urine was centrifuged to remove solid matter, acidified (pH 4) with acetic acid and washed several times with ether. It was then passed through a bed of activated charcoal (500 g.), which was afterwards washed with water (4 l.) to remove urea and inorganic salts, and then with aqueous 2N-NH_3 soln. (1·5 l.). The naphthalene metabolites were finally eluted with methanol (2 l.), and the eluate was evaporated to dryness under reduced pressure to a brown gum.

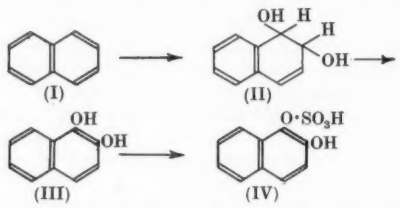
A cellulose-powder column was prepared: a mixture of *n*-butanol–*cyclo*hexane–water (13:8:1, by vol.) was added to cellulose powder (300 g., Whatman standard grade) to form a slurry, which was poured into a glass column (length 100 cm., diameter 8 cm.) fitted with a sintered disk as a support for the absorbant. The column was filled to the top with the solvent mixture and kept overnight to allow the cellulose powder to settle, after which solvent mixture (2 l.) was passed through the column at the rate of 100 ml./hr.

The gum containing the metabolites was dissolved in a little water and the solution was absorbed in cellulose powder (5 g.) which was packed in a layer on top of the cellulose in the column. The solvent mixture was passed through the column at 50 ml./hr., when a broad band which had a violet fluorescence under ultraviolet light was seen to form. The eluate was collected in 100 ml. fractions, which were separately evaporated to dryness under reduced pressure and examined by means of paper chromatography with the solvent systems already mentioned. The earlier fractions contained mainly *trans*-1:2-dihydronaphthalene-1:2-diol and were discarded. Later fractions, which corresponded to the elution of the fluorescent band, were evaporated to form light-brown gums or partially crystalline solids, all of which contained 1-naphthyl sulphate, together with the new metabolite. These gums were combined and dissolved in water (5 ml.) and 5N-NaOH (5 ml.) was added, followed by ethanol (50 ml.). The mixture was warmed until crystals began to separate, cooled to 0° and filtered. The filtrate was evaporated down to half volume on the steam bath and cooled to 0°, when more crystals separated. The combined solids were recrystallized from aqueous ethanol to yield a substance which was probably the disodium salt of 2-hydroxy-1-naphthyl sulphate as silky needles (980 mg., after drying at 100° and 0·2 mm., corresponding to about 2% of the naphthalene administered), which had an infrared spectrum (measured, as a mull in liquid paraffin, between 5 and 15 μ) identical with that of the substance obtained from the oxidation of 2-naphthol. It was converted as before into sodium 2-hydroxy-1-naphthyl sulphate (420 mg.), which formed plates from methanol–ether (Found: C, 45·4; H, 2·7; S, 12·0; Na, 9·0. Calc. for $C_{10}H_7O_5SNa$: C, 45·8; H, 2·7; S, 12·2; Na, 8·8%). The infrared spectrum (measured, as a mull in liquid paraffin, between 5 and 15 μ) was identical with that of the synthetic sodium salt, and the two salts had identical properties on paper chromatograms. When urines of the treated animals were examined on paper chromatograms with the solvent systems, the ester had somewhat higher R_F values than those obtained when the pure compounds were run (cf. Solomon, 1955; Corner & Young, 1954, 1955).

The ester was hydrolysed with 5N-HCl and the product was purified as before to yield 1:2-dihydroxynaphthalene, m.p. and mixed m.p. 101–102°. The diacetate formed prisms from ethanol, m.p. and mixed m.p. 108–109°. The mother liquors from the hydrolysis contained inorganic sulphate.

DISCUSSION

The structure of the new metabolite follows directly from its method of synthesis and from the fact that it gives 1:2-dihydroxynaphthalene and sulphuric acid on acid hydrolysis. The present work throws no light on the method of formation of the ester in the body but the work of Corner & Young (1955) and of Sato *et al.* (1956) indicates that 1:2-dihydronaphthalene-1:2-diol is an intermediate in its formation.



The metabolic pathway of naphthalene therefore involves oxidation to 1:2-dihydronaphthalene-1:2-diol (II). The diol (II) is then dehydrogenated to 1:2-dihydroxynaphthalene (III) which is then conjugated to yield the sulphuric ester (IV).

SUMMARY

1. The isolation of 2-hydroxy-1-naphthyl sulphate from the urine of rabbits dosed with naphthalene is described.

2. The ester was synthesized by the persulphate oxidation of 2-naphthol.

We thank Miss M. J. Allen for assistance with the animals, Dr S. F. D. Orr for the infrared spectra, Mr R. E. S. Prout for technical assistance and Mr P. Baker of Wellcome Research Laboratories for the micro-analyses. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

Boyland, E. & Solomon, J. B. (1956). *Biochem. J.* **63**, 679.
Corner, E. D. S. & Young, L. (1954). *Biochem. J.* **58**, 647.
Corner, E. D. S. & Young, L. (1955). *Biochem. J.* **61**, 132.
Desai, R. B. & Sethna, S. (1951). *J. Indian chem. Soc.* **28**, 213.
Fieser, L. F. & Fieser, M. (1939). *J. Amer. chem. Soc.* **61**, 596.
Fieser, L. F. & Peters, M. A. (1931). *J. Amer. chem. Soc.* **53**, 793.
Sato, T., Yamada, M., Suzuki, T., Fukuyama, T. & Yoshikawa, H. (1956). *J. Biochem., Tokyo*, **43**, 25.
Solomon, J. B. (1955). Ph.D. Thesis: University of London.

A Nitrogenous Derivative of Lactose from Lactating Rat Mammary Gland

By R. HEYWORTH* and J. S. D. BACON†
Department of Biochemistry, University of Sheffield

(*Received* 11 *October* 1956)

During an investigation of the synthesis of lactose by various preparations from rat mammary glands (see Heyworth & Bacon, 1955) a new substance was noticed on paper chromatograms of extracts made in the early stages of lactation. Shortly afterwards Caputto & Trucco (1952) reported the presence in such extracts of several oligosaccharides, one of which they suggested was a tetrasaccharide. We attempted the isolation of the substance, with the possibility in view that it arose as a by-product or intermediate in lactose synthesis. The idea that it was exclusively carbohydrate in nature was weakened by the discovery that acid hydrolysis only doubled its reducing power, and after the report by Leloir & Cardini (1953) that Caputto & Trucco's compound was a nitrogenous derivative of lactose we found that our own partially purified preparations also contained nitrogen. Although our method of purification differs considerably from that used by Trucco & Caputto (1954), the substance obtained resembles their 'neuramin–lactose' in many respects. Our compound, like 'neuramin–lactose' and 'compound E' (Caputto & Trucco, 1952), gives the direct Ehrlich reaction and a purple colour with Bial's reagent, which are characteristic of sialic acid (Blix, 1936) and neuraminic acid (Klenk, 1941). Although we normally used boiling water for its extraction, it may equally well be extracted with cold water. It therefore has a particular interest as a naturally occurring derivative of sialic acid of low molecular weight; hitherto sialic acid has been found only in mucins, glycoproteins and complex lipids.

The substance has no free amino group, and among the products of its alkaline degradation is a substance very similar to, if not identical with, 2-carboxypyrrole (Gottschalk, 1953, 1955*a*). It therefore resembles sialic acid more closely than neuraminic acid (methoxyneuraminic acid: Klenk & Faillard, 1954).

In this paper we describe the method of purification and some of the properties of the compound, including the products of its acid and alkaline degradation. A preliminary account has already been published (Heyworth & Bacon, 1954).

* Present address: Biochemistry Department, Royal National Orthopaedic Hospital, Stanmore, Middlesex.

† Present address: Department of Biochemistry, Macaulay Institute for Soil Research, Aberdeen.

EXPERIMENTAL AND RESULTS

Extraction and purification of the compound

Starting material. The substance was first observed on a paper chromatogram of an aqueous extract of rat mammary gland made at room temperature in the presence of $HgCl_2$ (see Heyworth & Bacon, 1955); benzidine–trichloroacetic acid (Bacon & Edelman, 1951) showed a spot of low R_F (about 0·03–0·06 in butanol–acetic acid–water, 4:1:5, by vol.; Partridge, 1948) of comparable intensity to the lactose present. Chromatography of extracts of glands at different stages of lactation showed that the compound of low R_F was present at 4, 5, 7, 10 and 12 days' lactation, but to a much smaller extent at 14 and 17 days. Glands of rats of 4–11 days' lactation were used for subsequent preparations.

The compound was not detected in preparations of guinea-pig mammary gland used for metabolic studies (Heyworth & Bacon, 1955), nor in a boiled and concentrated extract of guinea-pig glands (5 μl. applied to paper, corresponding to 7 mg. fresh wt. of tissue). Similarly, the compound was not detected in a boiled and deproteinized extract of a cow udder (10 μl. applied, corresponding to 8 mg. fresh wt. of tissue).

Extraction and deproteinization. The glands were chopped into boiling water and boiled for a further 10 min. to inactivate enzymes. The extract was cooled and blended, and the solid material separated in a refrigerated centrifuge. The hardened layer of fat was removed, and the residue resuspended in water and centrifuged again. The supernatants were collected and deproteinized; unless deproteinized the extract quickly blocked a charcoal–Celite column. Ethanol (0·5 vol.) and chloroform (0·25 vol.) were added, and the pH was adjusted to 4·5 with acetic acid. After being shaken vigorously the mixture was left overnight in the refrigerator. The white protein precipitate was removed by centrifuging, resuspended in ethanol–chloroform–water mixture and again centrifuged. The supernatants were concentrated under reduced pressure, with a water-bath temperature below 50°. Many extracts were divided into two parts (*a* and *b*, Table 1) before being chromatographed. A single preparation was made in which the initial extraction was carried out with cold water.

Chromatography on charcoal—Celite. This was carried out by gradient elution, as described by Bacon & Bell (1953) and Bacon (1954). Usually 40 g. of charcoal–Celite mixture (1:1) was packed in a glass tube of internal diameter 15 mm.

and eluted from a 500 ml. water reservoir into which 50% (v/v) ethanol was dropped to replace the effluent.

Columns containing Activated Charcoal (British Drug Houses Ltd.) were used for all the initial separations, and Active Carbon no. 130 (Sutcliffe, Speakman and Co. Ltd., Leigh, Lancs.) for the removal of impurities in the effluent from cellulose columns (Bacon, 1954). The columns were washed with about 1 l. of water overnight before the sugar mixture was added. This reduced the acid content of the effluents from B.D.H. activated charcoal some 40 times; the remaining acidity was neutralized with 0·1 N-NaOH. The addition of Na^+ ions increased the dry weight of the preparations from these columns by 1–4%.

The concentration of sugars in the effluent was sufficient for a direct test on paper chromatograms. In early experiments with B.D.H. activated charcoal there was a satisfactory separation of lactose and the unknown compound, which emerged at about 7 and 16% of ethanol respectively. Later batches of this charcoal failed to bring about any separation, but a sample of the same charcoal 'washed with acid' was found which reproduced the earlier results. The two charcoals were thereafter used in a two-step procedure, by which the two substances were first separated from other constituents of the extract, and then from one another.

Chromatography on cellulose powder. The method of Hough, Jones & Wadman (1949) was used, with the modifications described by Bacon (1954).

Evaporation of effluents. This was carried out at neutral pH, under reduced pressure below 50°, the residue being placed in an evacuated desiccator over H_2SO_4 or $CaCl_2$ at room temperature for determination of dry weight (Table 1) and for subsequent storage.

Types of preparation. Preparations used for analysis and investigation of chemical and physical properties were of two kinds: (i) those resulting from one or more fractionations on B.D.H. activated charcoal–Celite; (ii) preparations of type (i) further purified by partition chromatography on cellulose powder, followed by gradient elution from Active Carbon no. 130–Celite. Where the type of preparation is not specified it may be taken that both types possess the properties described. Both types showed only one spot on paper chromatograms sprayed with benzidine–trichloroacetic acid. (Here, and elsewhere, unless otherwise specified, the chromatograms were developed with *n*-butanol–acetic acid–water.) Type (ii) preparations showed only one spot, close to lactose, when run in ethyl acetate–pyridine–water (10:5:6, by vol.) at 30° (Trucco & Caputto, 1954); at 20–22° (solvent homogeneous) the spot was more compact and had an R_F value 0·7 times that of lactose.

Properties of the partly purified compound

Bial and Ehrlich tests. Bial's reagent (0·2 ml. of 0·25% orcinol, 0·01% of $FeCl_3$ in 12 N-HCl) gave no reaction with the compound (4 mg. in 0·2 ml.) in the cold, but on boiling gave a stable red colour.

Ehrlich's reagent [0·2 ml. of 3% (w/v) *p*-dimethylaminobenzaldehyde in 6 N-HCl] gave a green mixture with the compound (4 mg. in 0·2 ml.) in the cold and a red colour on heating.

Because of the acid content of these reagents it was not possible to spray them on to paper chromatograms and then heat them in a dry oven at 100°. However, it was found possible to steam the sprayed chromatogram. The steaming was carried out at atmospheric pressure on a wood and Perspex frame in an autoclave; the spots spread if too much water condenses on the paper.

On chromatograms of the preparations both the Bial's and Ehrlich's reagents gave purple spots corresponding with the sugar spot located by benzidine–trichloroacetic acid). The sugar and Bial-reacting spots also coincided on a chromatogram of the compound in *iso*butyric acid–ammonia (Krebs & Hems, 1953); (R_F 0·16; lactose, 0·20; galactose and glucose, 0·31). In this case the purple spot was not seen on steaming, but appeared after several hours at room temperature.

Absorption curve of the products of Bial's reaction. The reaction was carried out according to the methods of Klenk & Langerbeins (1941) and Werner & Odin (1952), with orcinol purified by treatment with charcoal in boiling

Table 1. *Yields, and reducing sugar and nitrogen contents of various preparations of the nitrogenous compound from rat mammary glands*

Type (ii) preparations were made from type (i) preparations of the same number. Preparation 7*a* (solid) was prepared by precipitation from a methanol solution with ether. For further details see text.

Tissue taken (g.)	Preparation (No.)	Preparation (Type)	Dry wt. (mg.)	Reducing sugar* (% dry wt.)	Nitrogen (% dry wt.)	Total nitrogen in sugar position† (%)	Ratio N:lactose
98	6*a*	(i)	27	31	—	—	—
	6*b*	(i)	41	43	3·2	—	1·8
109	7*a*	(i)	65	34	2·9	—	2·2
	7*b*	(i)	82	34	2·4	—	1·7
	7*b*	(solid)	12	41	2·2	—	1·3
73	9*a*	(i)	75	—	3·6	29	—
	9*b*	(i)	104	—	2·0	69	—
	9*a*	(ii)	12	27	1·2	82	1·2
105	11*a*	(i)	75	—	—	—	—
	11*b*	(i)	151	—	—	—	—
	11*a*	(ii)	22	23	1·2	—	1·2
105	12*a*	(i)	56	—	—	—	—
	12*b*	(ii)	—	—	—	—	—
	12*b*	(ii)	71	33	1·6	—	1·2

* Reducing sugar is calculated as though it were lactose. † On paper chromatograms.

benzene and subsequently crystallized from chloroform. With one type (ii) preparation the absorption curve coincided almost exactly with that given by Werner & Odin (1952) for sialic acid. Other preparations gave more indication of the inflexion near 650 mμ published by Trucco & Caputto (1954), though in no cases quite so pronounced. It seems possible that pentose, which has an absorption peak in this region after treatment under the same conditions, may be responsible for the inflexion.

Absorption curve of the products of the direct Ehrlich reaction. The reaction was carried out as described by Werner & Odin (1952), with *p*-dimethylaminobenzaldehyde purified by the method of Adams & Coleman (1944). The absorption curve was almost identical with that given by them for sialic acid, having maxima at 530 and 560 mμ, the latter somewhat greater.

Acid hydrolysis. In all cases after acid treatment of the compound chromatograms of the products sprayed with benzidine–trichloroacetic acid showed first the liberation of lactose and then the formation of glucose and galactose. [The untreated substance, like lactose, gave a blue–purple colour with the diphenylamine–aniline–phosphoric acid reagent (cf. Harris & MacWilliam, 1954).] Bial- and Ehrlich-reacting products appeared as purple spots on steaming and the same positions appeared as blue spots after several months' storage on chromatograms sprayed with benzidine–trichloroacetic acid or acid aniline phthalate (Table 2).

A hydrolysate (0·8 mg. in 0·1 ml. heated at 100° with 0·9N-H_2SO_4 for 10 min.) was diluted and passed through a column of De-Acidite E (Permutit Co. Ltd., Gunnersbury Ave., London, W. 4). The effluent was concentrated and applied to a chromatogram. After 90 hr. development separate spots of galactose and glucose were detected. No other reducing substances (Trevelyan, Procter & Harrison, 1950) and no hexosamines (Partridge, 1948) were detected in hydrolysates and there was no reaction on spraying with ninhydrin (0·1% in *n*-butanol) or bismuth potassium iodide (Bartley, 1954).

A chromatogram of preparation 9*b* (Table 1) contained 69% of the total nitrogen in the sugar position. On acid hydrolysis (0·3N-HCl, 100°, 10 min., Table 2) this nitrogen was redistributed, only 12% remaining in the original position (R_F 0·00–0·04), the remainder appearing from R_F 0·04 to 0·30. (The nitrogen-assay method is described below.)

Ratio of glucose:galactose. Samples of lactose (5·8 mg.) and of the compound (9·6 mg.) were each boiled in glass-stoppered tubes with 0·5 ml. of N-H_2SO_4 for 1 hr. The hydrolysates were cooled, diluted and passed through 5 g. columns of De-Acidite E. The effluents were concentrated to 0·5 ml. and applied to paper chromatograms (20 μl. along 7 cm. for estimation, two 5 μl. guide spots.). The chromatograms were developed with *n*-butanol–pyridine–water (6:4:3, by vol.) for 40 hr. The guide strips were sprayed with benzidine–trichloroacetic acid; only glucose and galactose were detected. The monosaccharides were eluted and the reducing powers measured with correction for paper blanks (Heyworth & Bacon, 1955). The ratio of the reducing powers of glucose:galactose both for lactose and the unknown compound was 1·2:1. It was concluded that there was an equimolecular ratio of the monosaccharide residues in the compound.

Reducing power. The lability of the compound to both acid and alkali made it difficult to decide whether a free reducing group was present. The following results are in favour of the presence of such a group:

(*a*) A chromatogram of the compound was sprayed with the benzidine–acetic acid reagent of Horrocks & Manning (1949); the formation of a brown spot on heating at 100° was as rapid as with glucose.

(*b*) Chromatograms of the compound dipped in silver nitrate solution in acetone and sprayed with ethanolic sodium hydroxide solution (Trevelyan *et al.* 1950) gave an immediate dark-brown spot.

(*c*) Evidence was obtained for the formation of an *N*-benzylglycosylamine derivative under the conditions of Bayly & Bourne (1953). In butanol–ethanol–water–ammonia (40:10:49:1, by vol.) lactose and the compound had R_F values 0·13 and 0·05, and the corresponding glycosylamines had R_F values 0·60 and 0·31 respectively. After spraying the chromatograms with 0·1% ninhydrin in butanol

Table 2. *Products of acid treatment of the nitrogenous compound*

R_F values in butanol–acetic acid–water from a number of experiments are given for spots reacting with benzidine–trichloroacetic acid at once (BT) or after storage (slow BT), with Ehrlich's reagent (E), or with Bial's reagent (BL).

Range of R_F value	Conditions of acid treatment: 0·3N-HCl; 80° 10 min.	0·3N-HCl; 80° 60 min.	0·3N-HCl; 100° 10 min.	0·5N-H_2SO_4; 80° 10 min.	0·5N-H_2SO_4; 80° 60 min.	N-H_2SO_4; 100° 3 min.*
0·0–0·1	0·06 (BT)	0·06 (BT)	0·05 (BT)	0·07 (BT)	0·07 (BT)	0·07 (BT)
0·1–0·2	0·11 (slow BT)	0·11 (slow BT)	—	—	—	—
	0·11 (BL)	0·11 (BL)	—	—	—	—
		0·16 (BT)	0·14 (BT)	—	—	—
	0·17 (slow BT)	0·17 (slow BT)	—	—	0·17 (BT)	0·19 (BT)
	0·17 (BL)	0·17 (BL)	—	—	—	—
0·2–0·3	—	—	0·24 (slow BT)	0·23 (BL)	0·23 (BL)	—
				0·25 (E)	0·25 (E)	0·27 (E)
		0·30 (BL)	—	0·27 (slow BT)	0·27 (slow BT)	0·30 (slow BT)
0·3–1·0	—	—	—	—	—	0·63 (E)

* In phenol–water: slow acid aniline phthalate, 0·43; Ehrlich, 0·42, 0·58, 0·70. In ethyl acetate–pyridine–water: slow acid aniline phthalate, 0·52; Ehrlich, 0·56.

and heating for 3 min. at 85° the derivatives appeared as yellow spots, which after 30 min. at room temperature changed to purple. Both the original sugars and the derivatives could be detected by spraying with benzidine–trichloroacetic acid. The greatest conversion of lactose into the benzylglycosylamine (about two-thirds) was obtained by heating the sugar on the chromatogram with benzylamine at 85° for 9 min. (instead of 5 min.). The conversion of the compound into the benzylglycosylamine under these conditions was, however, only about one-fifth.

When the compound and lactose were treated with benzylamine on chromatograms and these were developed in butanol–acetic acid, the acidic nature of the solvent caused a breakdown of the benzylglycosylamines and the original sugars appeared unchanged. This suggests that the reaction with benzylamine is reversible, as would be expected if the compound possesses a free reducing group.

The reducing power of the preparations under alkaline conditions was compared with that of lactose by the procedure of Nelson (1944) and Somogyi (1945). A boiling time of 15 min. was sufficient for complete reduction. The determinations were made in duplicate. The percentage of lactose in the compound was calculated on the assumption that the reducing power of the compound is equal to that of its lactose content. The results (Table 1) suggest that lactose accounts for about one-third of the dry weight of the type (ii) preparations.

Increase in reducing power on hydrolysis. The compound was hydrolysed by boiling with N-H_2SO_4 for 2 hr. The solution was cooled and neutralized with N-NaOH and reducing power measured by the Nelson–Somogyi procedure. The increases in reducing power in two experiments were 70 and 71% respectively of the reducing power before hydrolysis. A similar increase was found for the hydrolysis of lactose under these conditions.

Nitrogen determinations. Estimations of nitrogen were made on 4 mg. samples of the compound (in 0·5 ml.) by the micro-Kjeldahl procedure. A.R. sulphuric acid (1 ml.) and about 250 mg. of catalyst (5 g. of K_2SO_4: 1 g. of $CuSeO_3$) were added and the mixture was heated for 2 hr. in Pyrex tubes. Determinations and blanks were carried out in duplicate; the results are given in Table 1.

In order to determine how much Kjeldahl nitrogen was associated with the sugar-reacting component of the preparations, and the fate of this nitrogen after hydrolysis, estimations were carried out on strips cut across paper chromatograms.

The compound (about 8 mg. in 200 μl.) was applied along 7 cm. of the base-line of chromatograms. The papers were developed for short periods (5–15 hr.) with butanol–acetic acid, or propanol–ethyl acetate–water (7:1:2, by vol.). After drying, successive strips about 2 cm. deep were cut from the paper, and each was incinerated for about 3 hr.

In preparation 9*a* (Table 1) 29% of the nitrogen was associated with the sugar. After purification on a cellulose column and on Active Carbon no. 130 the percentage of nitrogen in the sugar position was 82, the remainder being distributed down the chromatogram in amounts no greater than the experimental variation of blank determinations.

Ratio of nitrogen: lactose. From the nitrogen and reducing-sugar estimations the ratio atoms of nitrogen: molecules of lactose was calculated for a number of preparations (Table 1). The preparations which had been purified on cellulose and Active Carbon no. 130 gave consistently the lowest ratio of 1·2:1. The evidence thus suggests that the ratio of nitrogen: lactose in the compound is 1:1. If the reducing power of the compound is less than that of the corresponding amount of lactose, then the ratio of nitrogen: lactose would be nearer to unity.

Tests for nitrogenous substances. (*a*) Biuret: a biuret test was negative.

(*b*) α-Amino acids: chromatograms were sprayed with 0·1% of ninhydrin in butanol (Consden, Gordon & Martin, 1944) and heated at 95° for a few minutes. Some preparations showed slight contamination with ninhydrin-reacting substances. Preparation 9*a* (Table 1) showed ninhydrin-positive spots on a butanol–acetic acid chromatogram at R_F 0·16, 0·20 and 0·63 (sugar at R_F 0·03). The ninhydrin-reacting substances were removed by chromatography on cellulose and Active Carbon no. 130.

(*c*) Determination of amino groups: no nitrogen was liberated by nitrous acid (Van Slyke, 1929) from a type (ii) preparation of the compound (6·2 mg. in 5 ml.).

(*d*) Basic nitrogen: chromatograms of the compound gave no reaction with the bismuth potassium iodide spray of Bartley (1954).

(*e*) Hexosamine and *N*-acetylhexosamine: chromatograms of the compound gave no reaction with the sprays of Partridge (1948). The conditions of the Morgan & Elson (1934) determination of acetylhexosamines (0·05 N-Na_2CO_3 at 100° for 5 min., and treatment with Ehrlich's reagent in the cold) gave no coloration.

Specific rotation. The specific rotations of four type (ii) preparations of the compound were measured in a 2 dm. polarimeter tube of about 3 ml. capacity. The values of $[\alpha]_D$ ranged from +6 to +22°.

Ultraviolet absorption. Paper chromatograms of the compound showed no darker areas under u.v. light (Chromatolite Lamp, Hanovia Ltd., Slough, Bucks). The absorption curve of an aqueous solution (preparation 7*b*, 1·2 mg./ml.) showed very little absorption between 420 and 340 mμ, rising to an optical density (1 cm. cell) of 0·3 at 250 mμ, with no peaks.

Phosphate, sulphur, halogen. Phosphate determinations (Berenblum & Chain, 1938) on some preparations of the compound showed traces of phosphate. A type (ii) preparation contained 0·01% of phosphate. No sulphur or halides were detected in Lassaigne sodium-fusion tests.

pH *Titration curve.* The compound (15 mg.) was dissolved in water (1 ml.) and titrated with HCl or NaOH from a 0·1 ml. ultramicroburette (The Emil Greiner Co., 20–26 N. Moore St., New York 13, U.S.A.). A Doran hanging-drop electrode was used for the measurement of pH. The curves obtained suggested the presence of a group with pK 2·2, and possibly another group with pK above 12. There was an indication that some change occurred at pH 12, rather more acid being required to reverse the titration than alkali originally consumed.

Paper electrophoresis. Solutions of the compound (3 μl. spots) were applied to a line drawn across the middle of 34 cm. × 5 cm. strips of Whatman 3MM paper. A 3 μl. spot of 2% (w/v) lactose was applied to each strip as a neutral marker. The apparatus used was the EEL paper-electrophoresis bath (Evans Electroselenium Ltd., Harlow, Essex). After a few hours with a current of 2 mA the strips were removed and allowed to dry, stretched horizontally on a stainless-steel frame. Lactose always moved towards the cathode by electro-endosmotic flow and its position was

taken as a base-line. The compound moved away 3·7 cm. towards the anode in 4 hr. with 0·02 M phosphate buffer, pH 6·5, indicating the presence of a negative charge at this pH. Similar results were gained in 0·01 N-HCl, and at pH 2·2 in phthalate buffer. A strip run at pH 7·0 in phosphate buffer and sprayed with Bial's reagent showed a purple spot in the same position as the sugar spot. Kjeldahl estimations showed a recovery of only 56% of the total nitrogen (preparation 7*b*, Table 1) in the Bial-spot position. Most of the remaining nitrogen (32%) was in the 'base' (lactose) position. In borate buffer at pH 9·6 the compound moved to the anode.

Infrared absorption spectrum. A type (i) preparation of the compound (19·8 mg.) was dissolved in methanol (3 ml.) and filtered through a small, fluted, hardened filter-paper into 50 ml. of ether. The ether was stirred continuously and a white flocculent precipitate appeared from each drop. The paper was washed with a little methanol. The ether was decanted, the precipitate washed and centrifuged twice with ether, and finally dried in a desiccator. Reducing-sugar estimation on the solid preparation gave a lactose content of 41%; the nitrogen content was 2·2% [Table 1: 7*b* (solid)]. These compared with analyses of 34% of lactose and 2·4% of nitrogen on the initial preparation. Thus it appears that some nitrogen-containing contaminants had been removed in the preparation of the solid. The ratio of nitrogen : lactose was 1·3 : 1 as against the earlier value of 1·7 : 1.

Drs E. J. Bourne and S. A. Barker kindly investigated the infrared spectrum of a Nujol (liquid paraffin) mull of this solid preparation and compared it with the spectra of lactose, lactose monohydrate, pyrrole and 2-carboxypyrrole. There were ranges of unresolved absorption by the solid preparation but the presence of lactose was supported by a number of absorption peaks. They reported the presence of a peak at a frequency of 872 cm.$^{-1}$ indicating an equatorial H, as in galactose or mannose but not in glucose, and a peak at 892 cm.$^{-1}$ which would be expected from a β-galactoside. There was less indication of the presence of a structure related to 2-carboxypyrrole. A pronounced peak at 1531 cm.$^{-1}$ in pyrrole and 1549 cm.$^{-1}$ in 2-carboxypyrrole was absent. An absorption peak at 1605 cm.$^{-1}$ was consistent with ionized COO^-.

Alkaline degradation. The R_F values on butanol–acetic acid chromatograms of the products of alkaline treatment are given in Table 3. Only with 0·025 N-NaOH was any of the original compound detected after heating. No other sugars were detected with the possible exception of traces of galactose. All the spots were red or purple. The 'slow' reaction with benzidine–trichloroacetic acid in this case took only about one day of storage.

Red or purple colours were given by 5 μg. spots of 2-carboxypyrrole with Bial's reagent (on steaming), with Ehrlich's reagent (in the cold), with benzidine–trichloroactic acid (immediately, on heating), with 0·1% ninhydrin (8 hr. at room temperature) and with bismuth potassium iodide (24 hr. at room temperature). The product of high R_F value (0·84) in the alkaline degradation products coincided with 2-carboxypyrrole. By visual comparison with an equivalent amount it was calculated that this product would account for 1% or less of the original preparation.

The other main final Bial-positive product of degradation was the compound with an R_F of about 0·40 (the figure of 0·33 was obtained with a chromatogram developed for only

Table 3. *Products of alkaline treatment of the nitrogenous compound*

R_F values in butanol–acetic acid–water from several experiments are given for spots reacting with the reagents listed in Table 2. The unchanged compound and other (BT)-reacting spots are not shown (see text). The reactions with Ehrlich's reagent (E) were given without heating of the sprayed paper. Spots reacting with bismuth potassium iodide (BiKI) or absorbing u.v. light (u.v.) are also indicated.

Range of R_F values	Conditions of treatment with NaOH at 80°											
	0·025 N	0·1 N		0·22 N	0·25 N							
	60 min.	15 min.	60 min.	60 min.	5 min.	10 min.	30 min.	40 min.	60 min.
0·0–0·1	—	—	—	—	—	0·08 (BL)	0·07 (E)	—	—
0·1–0·2	0·12 (BL)	0·13 (E)	—	—	—	0·11 (slow BT)	—	—	—
0·2–0·3	—	—	—	—	0·28 (E)	—	—	—	—
0·3–0·4	—	—	—	0·38 (E)	—	—	0·33 (E)	—	0·36 (BL)
				0·39 (slow BT)	—	—	0·33 (u.v.)	0·36 (BL)	0·39 (BiKI)
0·4–0·5	—	—	0·43 (E)	0·41 (u.v.)	—	—	—	0·40 (slow BT)	0·40 (E)
									0·40 (slow BT)
									0·43 (E)
0·5–0·6	—	—	—	—	—	—	—	—	—
0·6–0·7	—	—	—	0·60 (E)	—	—	—	—	0·66 (E)
0·7–0·8	—	—	—	—	—	—	—	—	—
0·8–0·9	—	—	0·85 (E)	0·85 (E)	—	0·88 (slow BT)	0·85 (E)	0·82 (BL)	0·82 (BL)
							0·85 (u.v.)	0·88 (slow BT)	0·83, 0·84 (E)
									0·87 (BiKI)

5 hr.). This product also gave a dark area when chromatograms were observed under u.v. light. In an estimation of nitrogen on the 5 hr. chromatogram, 50% of nitrogen appeared at R_F 0·10 and only traces at 0·33 and 0·85.

N-Acetylglucosamine heated at 80° with 0·25N-NaOH gave Ehrlich-reacting products with R_F 0·50, 0·61 and 0·74 at 5 min. (cf. Kuhn & Krüger, 1956). These had disappeared after 20 min. Glucosamine decreased to about one-tenth under the same conditions in 1 hr. With 0·025N-NaOH at 80° the two sugars decreased gradually over 1 hr.

Incubation with enzyme preparations. The compound was incubated at 37° and pH 5 with an almond-emulsin preparation, with a commercial lactase preparation (L. Light and Co. Ltd.) and with a dialysed aqueous extract of lactating rat mammary gland (Heyworth & Bacon, 1955), all of which hydrolysed lactose. Paper chromatography showed no change in the compound.

Incubation with respiring mammary-gland preparations. The compound (5 mg. in 4 ml.) was incubated with respiring slices, blends or mitochondrial suspensions from guinea-pig mammary gland (Heyworth & Bacon, 1955). There was no change in the appearance of the compound on paper chromatograms nor in the reducing power of the lactose position.

DISCUSSION

The partly purified compound here described was obtained in larger amounts per rat than the 'neuramin–lactose' of Trucco & Caputto (1954): 11 mg./rat, or 1·6 mg./g. of fresh tissue, as compared with the 0·4 mg./g. of fresh tissue reported by them. This may be due to the fact that our rats were at an earlier stage of lactation: 4–11 days, as against 15–19 days.

It is difficult to assess the purity of our preparations. Although no other carbohydrate was detectable by paper chromatography in the products of primary fractionation on charcoal, after purification on cellulose columns the material had a rather lower lactose content (as measured by reducing power) than Trucco & Caputto's. Assuming the non-lactose portion of the molecule to be similar to sialic acid ($C_{13}H_{23}O_{11}N$ or $C_{11}H_{19}O_{10}N$) one might expect the lactose to constitute between 49 and 53%; Trucco & Caputto (1954) found values of about 40–42%. It would probably be safe to assume that our best preparations were about 50% pure. On paper chromatography or electrophoresis a typical purified preparation showed a single spot which gave reactions both for reducing sugar and for sialic acid. After acid treatment this was no longer the case, the reducing sugar now having the R_F values of lactose, galactose and glucose (cf. Trucco & Caputto, 1954), and the sialic acid-like material migrating to other regions of the chromatogram. The quantitative data presented here are consistent with the hypothesis that the substance, like 'neuramin–lactose', is composed of one molecule of lactose and one of the nitrogenous sialic acid.

Our purified preparations were homogeneous in the ethyl acetate–pyridine–water solvent which Trucco & Caputto (1954) used to separate 'neuramin–lactose' from 'compound E', but we have not been able to decide whether it is identical with one or other of these (as seems likely), because Dr Trucco informs us that they have found considerable variations in R_F values, which they attribute to variations in the samples of commercial pyridine that they use.

The name 'neuramin–lactose' was fully justified by the evidence that was available to Trucco & Caputto in 1954, but it would seem from the fact that our compound yields 2-carboxypyrrole on alkaline degradation that it would be more correct to regard it as a sialic acid derivative (cf. Gottschalk, 1956). If, like bovine sialic acid (Blix, Lindberg, Odin & Werner, 1955), the lactose derivative of mammary gland has two acetyl groups, one more labile than the other, it seems possible that 'neuramin–lactose' and 'compound E' differ by one acetyl group. Unfortunately the greater part of the present work was completed in 1954, and we see no opportunity of investigating the acetyl content of our derivative, although it would clearly be very desirable to do so.

The recognition that the lactose derivative contains a substance of the sialic acid type does not help very greatly in understanding its structure, because the structure of sialic acid is still in doubt. The recent formulation of Gottschalk (1955*b*) (cf. Blix, 1956) would seem to provide only one functional group (>C=O) by which sialic acid could be attached to lactose, leaving the reducing group of the latter free, although there are evidently several hydroxyl groups through which the lactose could form a glycosidic linkage to the acid (Blix *et al.* 1955). The evidence presented here is not strong enough to decide between these two types of structure. Our evidence for a free reducing group could conceivably refer to the reducing properties of sialic acid itself [but see Gottschalk (1956) for the reducing properties of combined sialic acid] and the failure of three lactase preparations to attack the compound must be used with great caution as evidence against a lactoside structure, in view of the fact that *N*-acetyl-lactosamine is not attacked by emulsin nor by the lactase of *Escherichia coli* (Kuhn & Kirschenlohr, 1954).

The discovery by Dr M. Shilo that cell-free extracts of certain bacteria will liberate lactose from our compound should help considerably towards the identification of the non-lactose moiety, and its point of attachment (see Addendum).

As to the metabolic significance of the compound, Caputto & Trucco (1952) failed to observe any conversion of their substance into lactose, and our experience has been similar. The most promising system in which to test this further would be a

homogenate capable of lactose synthesis, such as those prepared by Reithel, Horowitz, Davidson & Kittinger (1952) and Kittinger & Reithel (1954). We have not been able to obtain such preparations (Heyworth & Bacon, 1955).

From the occurrence of sialic acid in conjugated proteins (including those of colostrum, Kuhn & Brossmer, 1954) one might argue that the presence of the lactose derivative in mammary gland is more likely to be connected with the elaboration of glycoproteins than with the synthesis of lactose. Nevertheless, it is tempting to speculate that the ability to synthesize lactose has been acquired by mammals by the modification of a synthetic mechanism which in other vertebrates is concerned only with the formation of mucins and other glycoproteins. The occurrence of lactose in plants (cf. Reithel & Venkataraman, 1956) is perhaps not relevant to this speculation.

SUMMARY

1. A nitrogenous derivative of lactose was extracted from the mammary tissue of rats of 4–11 days' lactation, and partly purified by gradient elution from charcoal–Celite and by partition chromatography on cellulose powder.

2. The compound gives purple colours characteristic of sialic acid when heated with the reagents of Ehrlich or of Bial, thus resembling the 'neuramin–lactose' of Trucco & Caputto (1954).

3. It further resembles 'neuramin–lactose' in the following: it yields lactose on acid hydrolysis, has a ratio of nitrogen:lactose (by reducing power) of 1·2:1, and has a strongly acidic group (pK 2·2), which the infrared absorption spectrum suggests is in the form of an ionized carboxyl group.

4. The compound differs from neuraminic acid in having no free amino group, and in yielding 2-carboxypyrrole on heating in alkaline solution.

5. It was not attacked by three lactase preparations, nor by respiring slices, homogenates and mitochondrial suspensions from guinea-pig mammary gland.

Part of this work was done with the technical assistance of Mrs B. Sutton. We are grateful to Dr R. E. Trucco for unpublished experimental details, and to Drs E. J. Bourne and S. A. Barker, of the University of Birmingham, for examining the infrared spectrum of one of our preparations. We also wish to thank Mr D. H. Williamson for synthesizing 2-carboxypyrrole, and Mrs E. E. Bacon for a gift of almond emulsin. We are indebted to the Medical Research Council for a scholarship (awarded to R. H.) and to the Agricultural Research Council for providing technical assistance during part of this investigation.

REFERENCES

Adams, R. & Coleman, G. H. (1944). *Org. Synth. (Coll.)*, 2nd ed., **1**, 214.
Bacon, J. S. D. (1954). *Biochem. J.* **57**, 320.
Bacon, J. S. D. & Bell, D. J. (1953). *J. chem. Soc.* p. 2528.
Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
Bartley, W. (1954). *Biochem. J.* **56**, 379.
Bayly, R. J. & Bourne, E. J. (1953). *Nature, Lond.*, **171**, 385.
Berenblum, I. & Chain, E. B. (1938). *Biochem. J.* **32**, 295.
Blix, G. (1936). *Hoppe-Seyl. Z.* **240**, 43.
Blix, G. (1956). *Acta chem. scand.* **10**, 157.
Blix, G., Lindberg, E., Odin, L. & Werner, I. (1955). *Nature, Lond.*, **175**, 340.
Caputto, R. & Trucco, R. E. (1952). *Nature, Lond.*, **169**, 1061.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Gottschalk, A. (1953). *Nature, Lond.*, **172**, 808.
Gottschalk, A. (1955*a*). *Biochem. J.* **61**, 298.
Gottschalk, A. (1955*b*). *Nature, Lond.*, **176**, 881.
Gottschalk, A. (1956). *Biochim. biophys. Acta*, **20**, 560.
Harris, G. & MacWilliam, I. C. (1954). *Chem. & Ind.* p. 249.
Heyworth, R. & Bacon, J. S. D. (1954). *Biochem. J.* **58**, xxiv.
Heyworth, R. & Bacon, J. S. D. (1955). *Biochem. J.* **61**, 224.
Horrocks, R. H. & Manning, G. B. (1949). *Lancet*, **1**, 1042.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1949). *J. chem. Soc.* p. 2511.
Kittinger, G. W. & Reithel, F. J. (1954). *J. biol. Chem.* **205**, 527.
Klenk, E. (1941). *Hoppe-Seyl. Z.* **268**, 50.
Klenk, E. & Faillard, H. (1954). *Hoppe-Seyl. Z.* **298**, 230.
Klenk, E. & Langerbeins, H. (1941). *Hoppe-Seyl. Z.* **270**, 185.
Krebs, H. A. & Hems, R. (1953). *Biochim. biophys. Acta*, **12**, 172.
Kuhn, R. & Brossmer, R. (1954). *Ber. dtsch. chem. Ges.* **87**, 123.
Kuhn, R. & Kirschenlohr, W. (1954). *Ber. dtsch. chem. Ges.* **87**, 1547.
Kuhn, R. & Krüger, G. (1956). *Ber. dtsch. chem. Ges.* **89**, 1473.
Leloir, L. F. & Cardini, C. E. (1953). *Annu. Rev. Biochem.* **22**, 182.
Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Reithel, F. J., Horowitz, M. G., Davidson, H. M. & Kittinger, G. W. (1952). *J. biol. Chem.* **194**, 839.
Reithel, F. J. & Venkataraman, R. (1956). *Science*, **123**, 1083.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Trucco, R. E. & Caputto, R. (1954). *J. biol. Chem.* **206**, 901.
Van Slyke, D. D. (1929). *J. biol. Chem.* **83**, 425.
Werner, I. & Odin, L. (1952). *Acta Soc. Med. Upsal.* **57**, 230.

ADDENDUM

The Breakdown of the Lactose Derivative by Bacteria

By M. SHILO*
Department of Biochemistry, University of Sheffield

(*Received* 11 *October* 1956)

In view of the lability of sialic acid under acid and alkaline conditions an enzyme preparation was sought that would split the linkage between lactose and the rest of the molecule, preferably without any further degradation of the latter. The action of micro-organisms and enzyme preparations from them was tested by incubating them with type (i) or (ii) preparations of the lactose derivative, and following the course of the reaction by paper chromatography.

RESULTS

Lactose-fermenting yeasts. Washed cells from cultures of lactose-fermenting yeasts supplied by Dr M. Aschner (*Oidium lactis*, *Candida subtropica*, *C. krusei* and *Torulopsis glabrata*) incubated with 20 mg./ml. of type (i) preparation at 30° did not attack the compound. However, bacterial contamination of some of these incubation mixtures led to its rapid breakdown. (For convenience, without prejudice to the final identification of the lactose derivative, these are referred to as 'neuramin–lactose-splitting bacteria.')

Isolation of neuramin–lactose-splitting bacteria. Solutions of type (i) preparations (20 mg./ml.) were incubated at 30° for 24–48 hr. under non-sterile conditions, with and without the addition of 1 % Tryptone (Oxo Ltd., Thames House, London, E.C. 4). From four such incubations active strains were isolated by plating on nutrient agar; all appeared to be identical. They were Gram-negative rods, and the colonies on nutrient agar were large, opalescent and strongly fluorescent under u.v. light. They were subcultured on nutrient agar (Marmite, 0·3 %; tryptone, 1 %; glucose, 1 %; K_2HPO_4, 0·3 %; NaCl, 0·5 %; agar, 2 %; water 100).

Action of resting cells. The bacteria were grown for 24 hr. on agar slopes of the above composition, washed off with 10 ml. of saline, centrifuged, washed with a further 10 ml. of saline and the centrifuged cells transferred to solutions (20 mg./ml.) of type (ii) preparations. Complete breakdown took place in 12–24 hr., yielding one substance with the R_F of lactose, and another spot which was Ehrlich-positive. The activity of a culture grown on nutrient-agar slopes to which 1 % of hog gastric mucin had been added was not noticeably greater.

Action of cell-free extracts. Cells were grown and harvested as described above. The thick paste of cells obtained on centrifuging was cooled to −15° and crushed in a Hughes press (1951). The cell-free extract was separated by centrifuging (about 10000 *g*); when stored frozen it retained its activity for several weeks. This extract (0·1 ml.) brought about the complete breakdown of type (ii) preparations (0·3 ml., 4 mg./ml.) in 4–9 hr. in the presence of chloroform, but many unwanted substances were simultaneously produced from the extract itself. All the side reactions detectable by the spraying agents used were eliminated by dialysis of the extract for 48 hr. against distilled water at 4°.

Properties of the breakdown products. The sugar released from combination had the same R_F as lactose in four solvent systems: *n*-butanol–acetic acid–water (4:1:5, by vol.), ethyl acetate–pyridine–water (2:1:2, by vol.; Zilliken, Smith, Rose & György, 1954), *n*-butanol–ethanol–water–ammonia (40:10:79:1; Bayly & Bourne, 1953) and ethyl acetate–*n*-butanol–water. The addition of a lactase preparation (L. Light and Co., Colnbrook, Bucks.) brought about the complete hydrolysis of the sugar and the appearance of two sugars with the R_F values of galactose and glucose.

The only other product detected was a Bial- and Ehrlich-positive spot, with an R_F rather greater than that of lactose in butanol–acetic acid–water (lactose has R_F about 0·08 on Whatman no. 1 paper), but hardly moving from the base line in ethyl acetate–butanol–water or in butanol–ethanol–water–ammonia. Its behaviour in butanol–acetic acid–water is thus distinguishable from that of acetylneuraminic acid (R_F 0·27 on Schleicher & Schüll 2043b paper, Klenk & Faillard, 1954), of lactaminic acid (R_F 0·85 with *sec.*-butanol, Kuhn & Brossmer, 1954), of gynaminic acid (R_F 0·66, Zilliken, Braun & György, 1955) and probably of methoxyneuraminic acid (R_F 0·72, Kuhn & Brossmer, 1954; 0·72, Zilliken *et al.* 1955; but 0·079, 0·084, Klenk & Faillard, 1954). This spot also gave a blue reaction after 3–4 weeks on chromatograms

* Present address: Laboratory for Microbiological Chemistry, Hebrew University, Hadassah Medical School, Jerusalem, Israel.

sprayed with benzidine–trichloroacetic acid. On some occasions the treatment with Bial's reagent seemed to show two very closely situated spots, but it is possible that this was a result of the migration which tends to occur during the steaming of the sprayed chromatogram.

Effect of pH. This was not investigated systematically, but the activity of cell-free extracts was distinctly greater at pH 5 than at higher pH values (up to 7·7). Sodium acetate–acetic acid buffer (0·05 M, pH 5·0) was added to incubation mixtures in the later experiments.

Neuramin–lactose splitting enzymes in other bacteria. (*a*) *Pseudomonas* spp. Cultures of *P. fluorescens*, *P. stuzeri*, and *P. pyocyaneus* were kindly provided by Dr S. R. Elsden of the Department of Microbiology of this University. Grown on the medium described above, and harvested and crushed in the same way, all three bacteria yielded cell-free extracts capable of splitting the lactose derivative, although more slowly than that from the strain first isolated; the extracts did not attack lactose.

(*b*) *Lactobacillus bifidus*, strain E, requiring the *bifidus* factor for growth, was very kindly provided by Dr D. J. D. Hockenhull, of the Glaxo Research Laboratories. It was grown in a liquid Boventer liver–infusion medium supplemented with 1% Bactopeptone (Difco Laboratories, Detroit, U.S.A.), 0·5% NaCl, 0·01% cystine, 1% lactose and 10% fresh deproteinized human milk (Tomarelli, Norris & György, 1949). The pH was adjusted to pH 7·4 and 400 ml. portions were sterilized in 500 ml. conical flasks in an autoclave (15 lb./in.^{2} for 15 min.). After inoculation with 5 ml. of a 24 hr. culture of the organism in the same medium, the flasks were incubated for 48 hr. at 37°. The cells were harvested and crushed as before.

Washed-cell suspensions and cell-free extracts split the lactose derivative, giving the Ehrlich- and Bial-positive substance described earlier, but no lactose; instead, monosaccharide appeared. The cell-free extract had lactase activity, which in the presence of *N*-acetylglucosamine produced a series of oligosaccharides similar to those described by Zilliken *et al.* (1954).

In the hope of inactivating the lactase the cell-free extract was dialysed for long periods against distilled water. When the lactose derivative was incubated with extracts dialysed for 18–24 hr., lactose was formed, indicating that the initial step in the action of extracts of *L. bifidus* is similar to that seen with the other bacterial extracts.

It would therefore seem that the capacity to split compounds like neuramin–lactose is of fairly widespread occurrence among bacteria.

SUMMARY

1. Washed-cell suspensions and cell-free extracts of several species of bacteria have been found to attack the lactose derivative from mammary gland described by Heyworth & Bacon (1957).

2. Two products of this action have been detected: a sugar having the chromatographic behaviour of lactose, and a sialic acid-like substance distinguishable from lactaminic acid (Kuhn & Brossmer, 1954) and gynaminic acid (Zilliken *et al.* 1955), and probably from acetyl- and methoxy-neuraminic acids (Klenk & Faillard, 1954).

I wish to thank those mentioned above for the gift of cultures, and the nursing staff of the Jessop Hospital for Women, Sheffield, for obtaining a supply of human milk. I am also grateful to the Humanitarian Trust for the award of a Fellowship.

REFERENCES

Bayly, R. J. & Bourne, E. J. (1953). *Nature, Lond.*, **171**, 385.
Heyworth, R. & Bacon, J. S. D. (1957). *Biochem. J.* **66**, 41.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Klenk, E. & Faillard, H. (1954). *Hoppe-Seyl. Z.* **298**, 230.
Kuhn, R. & Brossmer, R. (1954). *Ber. dtsch. chem. Ges.* **87**, 123.
Tomarelli, R. M., Norris, R. F. & György, P. (1949). *J. biol. Chem.* **181**, 879.
Zilliken, F., Braun, G. A. & György, P. (1955). *Arch. Biochem. Biophys.* **54**, 564.
Zilliken, F., Smith, P. N., Rose, C. S. & György, P. (1954). *J. biol. Chem.* **208**, 299.

The Dialysis of Brain Pyrophosphatase

By J. J. GORDON*
Research Department Bristol Mental Hospital, Fishponds, Bristol

(*Received* 20 *August* 1956)

It was previously reported (Gordon, 1953) that when rat-brain homogenates were dialysed against distilled water at 0° for 18 hr., about 90 % of their Mg^{2+} ion-activated pyrophosphatase activity was lost. The activity could be partly restored by addition of cysteine, which also had a slight activating effect upon the fresh, undialysed enzyme. This phenomenon does not appear to have been encountered with yeast pyrophosphatase, as the methods used in its purification have included dialysis (Bailey & Webb, 1944; Heppel & Hilmoe, 1951). The nature of the dialysis effect in brain pyrophosphatase was of some interest, for during attempts to purify the enzyme by methods involving fractional precipitation, it became desirable to remove ammonium sulphate by dialysis; this could not be done without practically complete destruction of the enzyme in every case. There appeared at first to be some possibility that the inactivation could be due to the loss of a diffusible cofactor (in addition to magnesium). Further work, however, led to the conclusion that the effect was due to the extreme lability of the enzyme under mildly acid conditions, the fall in pH at the membrane of the dialysis sac being apparently sufficient to cause the observed changes. The experiments which led to this conclusion are described in this paper.

EXPERIMENTAL

Enzyme preparation. Aqueous homogenates of rat brain were made by homogenizing the whole brain with ice-cold 0·9 % NaCl (2–3 ml.) in a Potter & Elvehjem (1936) apparatus, and diluting with water to approximately 10 mg./ml.

Activity measurements. These were carried out at 37° as previously described for fresh rat-brain homogenates (Gordon, 1953). Each estimation was carried out on at least two tissue concentrations (1·25–2·5 mg./ml.) at pH 7·4 in veronal buffer containing 0·008 M-$MgCl_2$. The total volume of the reaction mixture was always 4 ml., including the substrate (0·001 M sodium pyrophosphate). Appropriate blanks showed that none of the substances added to the reaction mixtures interfered with the Fiske–Subbarow phosphate estimation except 2:3-dimercaptopropanol (BAL). This substance was, however, readily removed by adding 1 ml. of $CuSO_4$ solution (1 %, w/v) with the trichloroacetic acid used as protein precipitant. The BAL was thereby precipitated as a copper complex and was removed together with the protein on filtration; control phosphate estimations then gave completely satisfactory results. Activities are expressed as m-moles of substrate/30 min./100 g. of tissue.

Dialysis against water. About 15 ml. of brain homogenate was placed in a Visking cellophan tube and dialysed against distilled water (6 l.) at 0° for 18 hr.

Dialysis against mixed resins. This was carried out as described by Gilbert & Swallow (1950), except that the experiments were done at 0°, and the dialysis time was 18 hr. The resins used consisted of Amberlite IR-100 (H^+ form) and Amberlite IR-4B (OH^- form).

RESULTS

As previously found (Gordon, 1953), when brain homogenates were dialysed at 0° against distilled water for 18 hr., at least 90 % of the Mg^{2+} ion-activated pyrophosphatase activity was lost, whereas storage at 0° for the same time resulted in only 15 % loss. Similar results were obtained when erythrocyte pyrophosphatase (a 1:100 dilution of washed, lysed rat erythrocytes) was subjected to the same treatment.

Activation by thiol compounds

It was confirmed that the addition of cysteine to the dialysed enzyme caused appreciable reactivation and was able also to cause slight activation of the undialysed enzyme. Two other thiol compounds, glutathione and BAL, showed similar effects. The relative efficiencies of the three thiol compounds are indicated in Table 1. The optimum reactivating concentrations differed greatly for the three compounds. BAL showed its maximum effect at a concentration approximately one-tenth of that of cysteine or glutathione, though the extent of reactivation was appreciably less than for cysteine. At optimum concentration, however, cysteine was the most effective in restoring activity, but still only reactivated the dialysed enzyme to the extent of about 42 %.

Failure of various other factors to activate the dialysed enzyme

The dialysed enzyme was completely uninfluenced by various amino acids which do not contain thiol groups, e.g. DL-alanine, L-glutamic acid, DL-aspartic acid, L-tyrosine, DL-tryphtophan, or

* Present address: Medical Research Council, Antibiotics Research Station, Clevedon, Somerset.

Table 1. *Activation of dialysed and undialysed brain pyrophosphatase by thiol compounds*

Activity of control = 31·0 m-moles/30 min./100 g.

Expt.	Enzyme	Thiol compound	Concn. (M)	Activity (% of control)
1	Undialysed	Cysteine	0·010	118·0
	Dialysed	Nil	—	12·9
	Dialysed	Cysteine	0·003	37·8
	Dialysed	Cysteine	0·006	49·6
	Dialysed	Cysteine	0·010	49·6
2	Undialysed	Glutathione	0·005	106·0
	Dialysed	Nil	—	10·7
	Dialysed	Glutathione	0·003	28·2
	Dialysed	Glutathione	0·005	28·2
3	Undialysed	BAL	0·0015	107·0
	Dialysed	Nil	—	10·3
	Dialysed	BAL	0·0005	25·7
	Dialysed	BAL	0·0015	25·7

DL-methionine, at concentrations similar to those used for the thiol compounds. Negative results were also obtained on adding either a concentrated form of the diffusate, or some enzyme preparation which had been inactivated by heating at 100° for 15 min.

The effects of $FeSO_4$, $ZnSO_4$ and $MnSO_4$ ($0{\cdot}04 \times 10^{-6}$ to $0{\cdot}1 \times 10^{-6}$) M were also tested, since it was reported by Pett & Wynne (1933, 1934) that bacterial pyrophosphatases were appreciably activated by low concentrations of Zn^{2+} and Fe^{2+} ions. King, Wood & Delory (1945) reported activation of erythrocyte acid phosphatase by Mn^{2+} ions, whereas Hove, Elvehjem & Hart (1940) found that purified alkaline phosphatase could be activated by low concentrations of Zn^{2+} or Mn^{2+} ions in combination with certain amino acids. In the present work on brain pyrophosphatase, completely negative results were obtained with Fe^{2+}, Mn^{2+} and Zn^{2+} ions, either alone or in combination with α-amino acids.

Since it appeared possible that the reactivating effect of thiol compounds was due to their reducing properties, those reducing agents used by Purr (1935) to reactivate papain after oxidative inactivation, i.e. NaCN (0·004 M), ascorbic acid (0·15 M) combined with $FeSO_4$ (4×10^{-8} M), and sodium thiosulphate (0·004 M), were examined. (It was confirmed that at these concentrations the substances did not inhibit the undialysed enzyme.) No reactivation of the dialysed enzyme was observed in any case. The above results made it seem unlikely that the dialysis effect could be due to the loss of a diffusible cofactor, and some other explanation of the phenomenon was sought. An experiment was carried out in which the enzyme was stored, but not dialysed, at 0° for 18 hr. in a cellophan sac. Under these conditions the enzyme lost only 30 % activity. Though this is greater than the loss which normally occurs on storage in the usual way at 0°, it was evident that the almost complete inactivation which takes place on dialysis is dependent on the actual process of dialysis, and not merely on the presence of the membrane; the inactivation could not, for instance, be due to the adsorption of a cofactor on the surface of the sac.

Lability of the enzyme under mildly acid conditions

The factors influencing the stability of the enzyme were next considered. Pillai (1938) concluded that pyrophosphatase in muscle is a very labile enzyme. Brain pyrophosphatase in acetone-dried powders is quite stable at 0° for several weeks (Gordon, 1950), but in aqueous dispersions of fresh tissue it is slightly inactivated (up to 15 %) on standing at 0° for 24 hr. Naganna (1950) found that erythrocyte pyrophosphatase was rapidly inactivated on incubation at 38° with acetate–veronal buffer at neutral pH. It seemed worth while to investigate the effect of pH on the stability of the enzyme at 0°, especially as a fall in pH may be expected during dialysis (Gilbert & Swallow, 1950).

Samples of a fresh enzyme preparation were adjusted to various pH values by adding 0·02 N-HCl, and allowed to stand at 0° for 18 hr. No buffering substances were added, but the pH was determined at the end of the storage period, and no perceptible change was found. After re-adjusting to pH 7·4 with dilute NaOH, the activities were measured as usual. (It was confirmed that NaCl in low concentrations did not inhibit the untreated enzyme.) The results showed that the enzyme was extremely labile in the presence of acid, losing 43 % of its activity at pH 5·9 and 73·6 % at pH 5·4. At pH 4·9, the enzyme was 85·3 % inactivated (comparable with that produced by dialysis). Similar experiments were carried out in which cysteine (0·01 M) was added to the enzyme after storage, and before proceeding with the activity

Table 2. *Effect of cysteine on brain pryophosphatase after exposure to acid*

Control activity without cysteine = 18·7; with cysteine = 23·9 m-moles of substrate/30 min./100 g.

pH	Inactivation (% of control activity) Without cysteine	With 0·01 M cysteine
7·4 (Control)	0	0
5·9	41·2	5·5
5·4	64·7	10·9
4·9	76·0	29·6

measurements; these results are given in Table 2. Cysteine appreciably reactivated the enzyme, as in the dialysis experiments.

The above results were consistent with the possibility that the dialysis effect could be due to changes in the pH of the homogenate during dialysis. An attempt was made to stabilize the pH by using 0·05 M acetate–veronal buffer (pH 7·4) instead of water as the dialysis medium, but under these conditions the enzyme was, as usual, inactivated to the extent of 90 %. The pH of a brain homogenate, dialysed in the usual way against distilled water, was tested before and after dialysis, but the pH fall was found to be only slight: from about 7·3 to 7·1. These results seemed surprising, but in view of the high acid-sensitivity of the enzyme it was still thought possible that more pronounced temporary changes of pH at the membrane during dialysis could be sufficient to inactivate the enzyme. To clarify this point, experiments were carried out in which the enzyme was dialysed against ion-exchange resins.

Dialysis against ion-exchange resins

The use of ion-exchange resins has been investigated by Gilbert & Swallow (1950), who were investigating methods of freeing protein and enzyme solutions from electrolytes by dialysis without denaturation of protein and inactivation of enzyme. They found that a urease solution, when dialysed through a cellophan sac against mixed resins, was inactivated; this was ascribed to a fall in pH on the membrane. On further investigation, these authors were able, with the mixed resins, to devise three methods of protecting the system against a fall in pH. In the present work it was thought that interesting data would be produced by the use of the Gilbert & Swallow (1950) techniques of dialysis. Experiments were carried out under the conditions described by these authors, with the slight modifications already referred to in the Experimental section.

The results of these experiments confirmed the conclusions already drawn, that the loss of enzymic activity on dialysis is due to a fall in pH value on the membrane. When the enzyme was dialysed against the mixed resins, with no precautions taken to prevent a fall in pH, it was inactivated to the extent of 92·5 %, as in ordinary dialysis. Gilbert & Swallow (1950) described three methods by which the fall in pH could be prevented, and each of these methods was applied. When the membrane was pretreated with *m*-phenylenediamine resin, only 33·5 % of the activity was lost. When the mixed resin system was pretreated with NaOH, only 3 % of the enzyme activity was lost, and when a layer of anion-exchange resin was interposed between the resin mixture and the membrane, there was 18·1 % inactivation. Each of these methods of preventing a fall in pH, therefore, gave also a substantial measure of protection against inactivation of the enzyme. The least protection was given by the resin-treated membrane; this confirms the observations of Gilbert & Swallow (1950), who indicated that this method was the least effective.

DISCUSSION

It is evident from the results described that dialysis effects are due to an actual denaturation of the enzyme, resulting from its extreme instability under slightly acid conditions. This ease of inactivation by acid may have some bearing on evidence already reported (Gordon, 1950) that active thiol groups are probably present in the enzyme. The essential thiol groups would be susceptible to spontaneous oxidation under various conditions; this apparently takes place very slowly even at 0° in neutral solution, and an acceleration of the process may be brought about by alteration in the pH. The partial reversal of the effect by thiol compounds is in agreement with such a mechanism, though it is difficult at present to see why the other reducing agents tried did not have some effect too. However, the results on the whole are consistent with the previous conclusion that brain pyrophosphatase probably has active thiol groups in its structure.

The observations reported here are also of interest with reference to the important problem of purifying the enzyme. Repeated attempts at purification have so far met with only slight success, and the readiness with which inactivation can take place imposes serious limitations on the kind of processes which can be applied. The stabilization of pH, and probably the necessity for operating under ice-cold conditions, are two important factors to be taken into account in devising suitable methods for the purification of the enzyme, the achievement of which will be necessary in order both to investigate more conclusively the chemical nature of this interesting system, and to attempt to elucidate its role in the metabolism and function of the brain.

SUMMARY

1. Brain pyrophosphatase is readily inactivated on standing at 0° in slightly acid solution.

2. The fall in activity which takes place when the enzyme is dialysed is not observed when the dialysis is carried out under conditions in which the pH is prevented from falling.

I wish to record my thanks to Mr R. J. Everson for valuable technical assistance.

REFERENCES

Bailey, K. & Webb, E. C. (1944). *Biochem. J.* **38**, 394.

Gilbert, G. A. & Swallow, A. J. (1950). *Biochem. J.* **47**, 502.

Gordon, J. J. (1950). *Biochem. J.* **46**, 96.

Gordon, J. J. (1953). *Biochem. J.* **55**, 812.

Heppel, L. A. & Hilmoe, R. J. (1951). *J. biol. Chem.* **192**, 87.

Hove, E., Elvehjem, C. A. & Hart, E. B. (1940). *J. biol. Chem.* **134**, 425.

King, E. J., Wood, E. J. & Delory, G. E. (1945). *Biochem. J.* **39**, xxiv.

Naganna, B. (1950). *J. biol. Chem.* **183**, 693.

Pett, L. B. & Wynne, A. M. (1933). *Biochem. J.* **27**, 1660.

Pett, L. B. & Wynne, A. M. (1934). *Biochem. J.* **28**, 365.

Pillai, R. K. (1938). *Biochem. J.* **32**, 1087.

Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.

Purr, A. (1935). *Biochem. J.* **29**, 13.

Potassium Uptake by *Alcaligenes faecalis*

By H. A. KREBS, R. WHITTAM and R. HEMS

Medical Research Council Unit for Research in Cell Metabolism, Departments of Biochemistry, Universities of Sheffield and Oxford

(*Received* 15 *August* 1956)

In experiments on the metabolism of the bacterium *Alcaligenes faecalis* it was noted that the capacity to respire is much reduced, often to almost zero, on washing the cells with distilled water, but is restored on addition of yeast extracts. The essential component of yeast extracts was found to be potassium. This organism differs from many others in that it can lose its intracellular potassium readily to the medium if the potassium concentration of the latter is low. The cells can recover potassium if its concentration in the medium is raised. This recovery is in part due to active transport, depending on respiration, but appreciable amounts of potassium can be taken up against concentration gradients without a supply of energy. This paper is concerned with a study of the potassium distribution between cells and medium under a variety of experimental conditions.

EXPERIMENTAL

Growth conditions. The basal medium contained 1% (w/v) of Difco peptone, 0·5% of Difco yeast extract, 1% (w/v) of casein hydrolysate (Pronutrin or Tryptone), and 1% (w/v) of glucose. In some of the later experiments glucose was omitted when it had been found that it was not oxidized. The pH was adjusted with NaOH to 7·0. The inoculum consisted of a 3–5 ml. liquid subculture of the same medium. Roux bottles containing 100 ml. of medium and 2% of agar were incubated at 30° for about 18 hr. The cells were washed on the centrifuge with three portions of water. After washing, the cells from one Roux bottle were suspended in 15 ml. of water and stored at +2°. This suspension contained generally 11–12 mg. of dry cells/ml. In the later experiments agar was omitted to avoid contamination of cell suspension with pieces of agar gel, and in order to reduce the path of diffusion of oxygen 50 ml. of medium was used/Roux bottle.

Organisms. Three strains of *A. faecalis* were examined. They were: strains 415 and 1347 of the National Collection of Type Cultures; and strain 19 obtained from the Department of Bacteriology, University of Sheffield. The organism is a Gram-negative rod incapable of fermenting sugars. Unless otherwise stated strain 1347 was used.

Ionic distribution. The distribution of potassium and sodium between medium and cells was determined as follows. At the end of the incubation period the suspension was thoroughly shaken and 3 or 5 ml. of the suspension was transferred to a weighed centrifuge tube of 6 ml. capacity. If the conditions of incubation were aerobic the suspension was gassed for 10 min. with oxygen to prevent anaerobic conditions in subsequent manipulations and the suspension was then spun for 10 min. in a refrigerated centrifuge at 20 000 *g*. As much as possible of the supernatant was removed with pipettes or by decanting and collected for analysis. The amount of cell residue was ascertained by reweighing the centrifuge tubes with the residue. The cell volume was calculated on the assumption (based on measurements) that the specific gravity of the residue was 1·017 27°). The specific gravity of the standard medium was 1·011. Examples of the calculations are contained in Table 3.

Fo analysis by flame photometry the supernatant was diluted usually to 10 ml., but less if the amounts of potassium or sodium were small. The residue was mixed with 0·05 ml. of conc. HNO_3 and placed for 30 min. in a boiling-water bath. This resulted in a clear solution which was diluted to a known volume, usually 10 ml. The diluting material

Table 1. *Effects of potassium chloride, yeast extract and ashed-yeast extract on the respiration of washed cells of* Alcaligenes faecalis, *strain* 1347

Cells were stored for 6 days at 2° and incubated at 30°; substrates, 0·005 M; KCl, 0·00125 M. Yeast ash refers to a quantity corresponding to the un-ashed material; sodium phosphate buffer, pH 7·4, 0·04 M; 3 mg. (dry) cells; total volume, 4 ml.; O_2.

		$-Q_{O_2}$			
Substrate	Period of observation (min.)	No addition	KCl	0·4 ml. of Difco yeast 1% (w/v)	0·4 ml. of yeast ash
L-Glutamate	40–60	10	78	148	80
	100–120	21	93	222	88
Pyruvate	40–60	9	26	124	26
	100–120	8	27	190	28

Table 2. *Effect of* K^+ *ions on the respiration of strain* 1347 *in the presence of various substrates*

Substrate concentration, 0·0125 M. Q_{O_2} calculated for 40–60 min. period; 2·85 mg. of (dry) cells was present in a total volume of 4 ml. Sodium phosphate buffer, pH 7·4, 0·04 M. Cells were 48 hr. old. Acidic substances were added as sodium salts.

	$-Q_{O_2}$	
Substrate added	Without added KCl	With 0·008 M-KCl
L-Glutamate	43	132
Acetate	8	74
DL-Lactate	24	153
Succinate	10	101
Pyruvate	24	127
Fumarate	9	58
Glycerol	3	22

included in both cases sodium and potassium for internal compensation.

Sodium and potassium were determined by flame photometry. In the earlier experiments the apparatus of Domingo & Klyne (1949) was used, in the later ones a Beckman spectrophotometer with the flame-photometry attachment 9200 and the photo-multiplier 4300. Chloride was determined potentiometrically (Sanderson, 1952).

The cell volume was ascertained by centrifuging a suitable amount (1 ml.) of suspension, the volume depending on the cell concentration, in a 'haematocrit', the capillary part of which had a diameter of about 1 mm. Centrifuging was continued at 20000 *g* until the volume was constant (usually about 30 min.).

Incubations. Washed suspensions were shaken in conical Warburg vessels under the conditions stated in the tables. The centre well usually contained 0·2 ml. of 2 N-NaOH. The oxygen consumption was measured in most aerobic experiments.

RESULTS

General metabolic properties of the organism

Washed suspensions of this organism showed a very low oxygen uptake on addition of amino acids, lactate, pyruvate, acetate or some intermediates of the tricarboxylic acid cycle [Q_{O_2} (O_2 used/mg. dry wt./hr.) = −10 to −20 at 30°] but respiration is very rapid (Q_{O_2} up to −250) when complex media such as yeast extracts or casein hydrolysate are added. Ashed-yeast extract, added together with glutamic or pyruvic acid, also increased the rate of oxidation but less effectively than un-ashed-yeast extract. Potassium chloride or other potassium salts have the same effect as the ash, which shows that potassium is an active principle of yeast ash. The effects of potassium chloride, yeast extract and ashed-yeast extract are shown in Table 1. The increase due to potassium was three- to eight-fold; yeast extract caused a further large rise which was much more marked with pyruvate than with glutamate.

The effect of potassium in the presence of various substrates is shown in Table 2. Every oxidizable substrate tested showed the effect though its magnitude varied from substrate to substrate. The effect of potassium chloride was larger and the effect of yeast extract smaller in fresh cells than in old cells, indicating that the factors which limit respiration change in the course of ageing.

Repeated washing on the centrifuge had no major effect on the effects of potassium chloride and yeast extract, one washing being almost as effective as three washings in producing cells which respond to the addition of potassium chloride and yeast extract. The yield of cells was slightly (20%) increased when the medium was supplemented by 0·5% potassium chloride. Cells grown in the presence of 0·5% potassium chloride showed the same response of respiration to potassium chloride after washing.

The same effect was shown by potassium sulphate, potassium chloride, and potassium phosphate when added in equivalent quantities. The effect reached a maximum when the concentration of K^+ ions in the medium was about 0·002 M. Salts of lithium, sodium, ammonium, magnesium, calcium and barium had no effects. A commercial sample of rubidium chloride (possibly containing potassium chloride) was almost as active as potassium chloride. Caesium chloride (analytical reagent) had a slight effect.

The pH optimum of the respiration with glutamate as substrate was between pH 7·4 and 7·7. At pH 6·2 the respiration was about 50 % of the maximum value.

Washed suspensions did not oxidize glucose, ethanol, citrate or α-oxoglutarate. When glutamate was the substrate α-oxoglutarate did not appear in the medium.

As shown in Table 1, yeast extracts stimulate the respiration more than the equivalent quantity of yeast ash or potassium chloride. Many substances known to be present in yeast extracts were examined for their stimulating effect on respiration, but no pure substance or combination of pure substances was found to replace these extracts fully. Substances tested included a mixture of pantothenate, pyridoxine, riboflavin, biotin, nicotinic acid, thiamine, cocarboxylase and inositol, together with mixtures of synthetic amino acids, glucose, pyruvate and other oxidizable substances. Lipoic acid and glutathione were also inactive.

Potassium and sodium content of strain 1347. Data on the distribution of potassium and sodium between medium and cells harvested after 16 hr. are shown in Table 3. The standard medium without agar was used. For the calculation of the intracellular sodium and potassium it had to be borne in mind that the centrifuged cells were 'contaminated' with medium, partly due to the impossibility of decanting the supernatant quantitatively and partly due to incomplete 'packing' of the cells. No correction was made for incomplete packing. The contamination with medium was corrected for on the basis of the haematocrit value (which supplied the volume of packed cells) and the analytical data for the medium. As seen in Table 3, the ratio intracellular potassium/extracellular potassium was about 7, the ratio intracellular sodium/extracellular sodium about 0·7. These figures are based on the assumption that potassium and sodium are evenly distributed throughout the cell. The actual potassium and sodium spaces are likely to be smaller than the cell volume and the real concentrations of potassium and sodium in the intracellular fluid may therefore be greater than calculated. This would increase the above ratios and make that for sodium nearer to 1.

Potassium content of washed cells after incubation. Cells of the culture used for the preceding experiment were washed twice with water on the centrifuge. The sediment was suspended in 1 ml. of water and divided into equal parts. One 0·5 ml. portion (containing 18·65 mg. of dry bacteria) was incubated at 15° with 3·5 ml. of fresh culture medium, the other was analysed for potassium. Incubation was for 1 hr. in Warburg flasks shaken in 100 % oxygen. The suspension absorbed about 600 μl. of oxygen in 60 min. The potassium analysis at the end of the incubation gave the following value: potassium in medium, 157 μg./ml.; potassium in cells, 1530 μg./ml. The corresponding values before incubation, i.e. on the medium and on the second 0·5 ml. portion of cells, were: potassium in medium, 202 μg./ml.; potassium in cells, 145 μg./ml. The data indicate that cells lose potassium on washing with water, but recover it on aerobic incubation in the nutrient medium. The level attained was of the same order as that found in freshly harvested cells (see Table 3).

Effect of external potassium level on the intracellular potassium concentration. Cells were washed twice with water and suspended in a small volume of water. Warburg cups contained 0·5 ml. of this cell suspension (16·4 mg. dry wt. of cells, 152 μl. volume wet), 1 ml. of 0·5 M-triethanolamine buffer, pH 7·4 (prepared by mixing 100 ml. of M-triethanolamine,

Table 3. *Distribution of potassium and sodium in cells of strain* 1347 *after growth for* 16 *hr. at* 30°

Duplicate analyses; for experimental conditions see text.

	Potassium		Sodium	
Volume of cells and medium centrifuged (ml.)	5·0	5·0	5·0	5·0
Volume of supernatant collected (ml.)	4·7	4·8	4·8	4·8
Weight of residue (mg.)	81·5	65·2	81·4	105·1
Volume of residue (μl.)	80·7	64·5	80·5	104·1
Volume of packed cells in residue (μl.)	30·9	30·9	30·9	30·9
Analyses				
K or Na/ml. of supernatant (μg.)	**220**	**216**	**317**	**318**
K or Na in total residue (μg.)	58·2	54·8	23·2	29·0
K or Na in medium of residue (calculated) (μg.)	11·0	7·26	15·7	23·1
K or Na in cells of residue (μg.)	47·2	47·5	7·5	5·9
K or Na/ml. of cells (μg.)	**1525**	**1540**	**243**	**191**

Determination of cell volume in suspension

1·012 ml. of suspension gave a haematocrit value of $\left\{\begin{matrix}6{\cdot}21\,\mu l.\\6{\cdot}16\,\mu l.\end{matrix}\right\}$ av. 6·185 μl.

Ratio dry wt./wet wt.; 5 ml. of suspension, spun and washed once, left 3·35 mg. dry wt., 30·9 mg. wet wt.

68 ml. of N-HCl and 32 ml. of water), 0·4 ml. of 0·1 M-sodium acetate, 0·1 ml. of 0·02 M-sodium fumarate and 2 ml. of potassium chloride solution of varying strength. Triethanolamine buffer, rather than a buffer containing a metallic cation, was used in order to keep the potassium or sodium concentration low. The temperature of incubation was 15°. This lower temperature was chosen because considerable quantities of cells were needed for the determination of intracellular potassium, and oxygenation of these quantities is difficult at higher temperatures. The gas phase was 100% oxygen. The data on the concentrations of external and internal potassium are given in Table 4. For comparison, data on the effect of Difco yeast extract are also given. The intracellular potassium level rose on incubation from 143 μg./ml. to about 1000 μg./ml. The intracellular level reached was independent of the external potassium concentration within the range tested. Difco yeast extract increased the oxygen uptake, but decreased the intracellular potassium level.

Another experiment at lower external potassium levels is shown in Table 5. The conditions were the same as in the previous experiment except that glucose was omitted from the growth medium. The intracellular potassium level reached in this experiment was lower than in the previous one. Whether this was connected with the absence of glucose from the growth medium remains to be investigated. The intracellular level was independent of the external level until the latter fell below 10 μg./ml. The ratio intracellular potassium/extracellular potassium rose as the external potassium concentration fell and was in the region of 100 at the lowest external concentration.

As shown in the last column of Table 5, potassium was also taken up anaerobically, but the internal level reached was about 30% lower than under the same conditions aerobically.

Further data on the anaerobic uptake of potassium at varying potassium concentrations in the medium are given in Table 6. The cells (grown in the absence of glucose) were added to the remainder of the solution from the side arm of the manometer cup after the air had been replaced by nitrogen and any remaining traces of oxygen had been absorbed by the yellow phosphorus. Considerable amounts of potassium were again taken up anaerobically against concentration gradients, but less than aerobically. The last two horizontal rows show the recovery of the potassium originally present and represent a check on the reliability of the procedure.

Effect of the constituents of suspending medium on the concentration gradient of potassium. Table 7 shows that at comparable external potassium concentrations much higher intracellular concentrations were obtained in the presence of the growth medium than in a simple medium containing oxidizable substrates. The concentration gradients were roughly twice as great in the presence of growth medium, aerobically as well as anaerobically. In order to

Table 4. *Effect of varying potassium concentrations in the medium on the level of intracellular potassium*

Dry cells (16·4 mg.) were present in a total volume of 4 ml. Incubation was for 1 hr. at 15°. For full details see text. The data refer to concentrations after incubation; the initial intracellular potassium concentration was 143 μg./ml.

Cup no.	1	2	3	4
KCl included in 4 ml.	2 ml. of 0·115%	2 ml. of 0·330%	2 ml. of 0·66%	2 ml. of 0·115% 2 mg. of Difco yeast
K in supernatant (μg./ml.)	48·5	109·2	236	65·2
K in cells (μg./ml.)	1060	928	1195	765
Ratio: $\frac{\text{intracellular K}}{\text{extracellular K}}$	22	8·5	5·1	11·7
O_2 uptake in 50 min. (μl.)	479	481	463	630

Table 5. *Effect of varying potassium concentrations in the medium on the level of intracellular potassium*

Dry cells (19·76 mg.) were present in 4 ml. of suspension. Incubation for 1 hr. at 15°. Initial intracellular potassium concentration, 120·1 μg./ml.

Cup no.	1	2	3	4	5
KCl included in 4 ml.	2 ml. of 0·115%	2 ml. of 0·0575%	2 ml. of 0·029%	2 ml. of 0·00143%	2 ml. of 0·0575%
Gas	O_2	O_2	O_2	O_2	N_2
K in supernatant (μg./ml.)	57·4	26·5	12·3	4·55	29·9
K in cells (μg./ml.)	567	590	553	459	429
Ratio: $\frac{\text{intracellular K}}{\text{extracellular K}}$	9·9	22·2	45·0	101	14·8
O_2 uptake in 50 min. (μl.)	356	351	343	342	0

examine the effects of the constituents of the growth medium, cells were incubated with modified media (Table 8). Omission of glucose or peptone or casein hydrolysate had no major effect on the concentration gradient.

In other experiments the effect of Difco yeast extract at a final concentration of 0·05 % was tested, with 0·01 M-sodium acetate and 0·0005 M-sodium fumarate as substrates. Aerobically the yeast extract increased the rate of oxygen consumption by about 25 % but there was no effect on the concentration gradient of potassium aerobically or anaerobically. Addition of sodium chloride (0·066 M) to the medium, on the other hand, caused a substantial uptake of potassium by the cells and a slight uptake of sodium (Table 9). After incubation in the presence of sodium chloride the potassium gradient was 1:47 in favour of the cells, whereas the sodium gradient was 1:18·5 in favour of the external medium.

Movements of other ions. The uptake of potassium must be accompanied by other movements of ions to maintain electroneutrality. Either an equivalent cation must migrate in the opposite direction, or an equivalent of an anion must move together with potassium. Experiments were set up in which the

Table 6. *Effect of potassium concentration of external medium on internal potassium level under anaerobic conditions*

The side arm of each cup contained 1 ml. of washed cells in water (23·8 mg. dry wt.); main compartment contained 1 ml. of 0·5 M-triethanolamine buffer, pH 7·4; 0·4 ml. of 0·1 M-sodium acetate; 0·1 ml. of 0·02 M-sodium fumarate and varying quantities of 0·23 % KCl solution. Total volume, 4 ml., made up with water. Incubation for 1 hr. at 15°.

Cup no.	1	2	3	4	5
0·23 % KCl added (ml.)	0·1	0	0·025	0·1	0·4
Gas	O_2	N_2	N_2	N_2	N_2
O_2 used (μl.)	510	0	0	0	0
K in supernatant (μg./ml.)	22·7	3·76	10·5	31·3	123
K in cells (μg./ml.)	515	176	214	306	472
Total K added (cells + KCl) (μg.)	159	37·9	68·1	158	518
K recovered (μg.)	172	41·9	73·8	166	550

Table 7. *Effect of oxygen on distribution of potassium in the presence and absence of growth medium*

Each cup contained 0·5 ml. of cell suspension (19·1 mg. dry wt.) in the side arm; main compartment contained 0·4 ml. of 0·1 M-sodium acetate; 0·1 ml. of 0·02 M-sodium fumarate and items shown below. Incubation for 1 hr. at 15°.

Cup no.	1	2	3	4
Main compartment ...	0·12 ml. of KCl 1·15 % (w/v) 2·9 ml. of H_2O	3 ml. of growth medium	0·131 ml. of KCl 1·15 % (w/v) 2·9 ml. of H_2O	3 ml. of growth medium
Gas	O_2	O_2	N_2	N_2
O_2 used in 50 min. (μl.)	341	633	0	0
K in supernatant (μg./ml.)	160	157	193	183
K in cells (μg./ml.)	1050	1800	633	976
Cell volume (μl.)	99·5	82·3	99·3	79·8
Ratio: $\frac{\text{intracellular K}}{\text{extracellular K}}$	6·6	11·5	3·27	5·3
K added (μg.)	754	769	820	769
K recovered (μg.)	730	765	823	788

Table 8. *Distribution of potassium between cells and medium under varying conditions*

Growth medium: 1 % of casein hydrolysate; 1 % of Difco peptone; 1 % of glucose; 0·5 % of Difco yeast extract; pH was adjusted to 7·0 with NaOH. Cells, 0·5 ml.; 3·5 ml. of medium. Incubation for 60 min. at 15° in O_2.

Expt. no.	Suspension medium	K (μg./ml.) Medium	K (μg./ml.) Cells	Ratio: $\frac{\text{K in cells}}{\text{K in medium}}$
1	Growth medium (cells 4 days old)	195	1145	5·9
2	Growth medium (fresh cells)	194	1775	9·2
3	Growth medium (cells 2 days old)	173	1440	8·3
	Same without glucose	136	1020	7·5
	Same without peptone	112	1030	9·2
	Same without glucose and peptone	118	1045	8·9
	Same without casein hydrolysate	128	1010	7·9

movements of potassium, chloride and sodium were measured. Cell suspension (4 ml.) containing 76·8 mg. of dry cells was shaken for 15 min. at 22° with 4 ml. of 0·0575% potassium chloride. No substrate was added. The potassium concentration of cells rose from 2·7 to 13·1 mM but the chloride concentration did not increase; in fact it showed a small decrease from 1·4 to 0·85 mM. There was a small drop in the sodium concentration from 2·67 to 0·70 mM. Thus rather less than 20% of the potassium uptake could be accounted for by an exchange with sodium. The remainder of the potassium uptake was not accounted for by the movements of other ions. Tests in similar experiments showed no change in acidity of the medium, indicating that potassium is not exchanged for H^+ ions (see Conway & O'Malley, 1946). Nor was there a change in the overall concentrations of nitrogenous substances, which makes an exchange for amino acids unlikely.

Another experiment in which sodium glutamate was added as a substrate aerobically and anaerobically gave similar results (Table 10). Again the increase in the intracellular concentration of potassium was not accompanied by an uptake of chloride or by a loss of sodium. On the contrary sodium was taken up by the cells, presumably together with the glutamate added to the medium.

Exchange of potassium between cell and medium. In order to obtain information on the nature of the forces by which the concentration gradients of potassium between cells and medium are maintained ^{42}K was added to a suspension of cells in which a steady-state concentration gradient had been established. The specific activity of the potassium in cells and medium was measured after further incubation both aerobically and anaerobically. Data obtained from this kind of experiment indicate whether the intracellular potassium is firmly bound or in rapid equilibrium with K^+ ions; $^{42}K_2CO_3$ neutralized with HCl served as a source of ^{42}KCl. The quantity of ^{42}K added (0·43 mg. in 0·5 ml.) did not cause a major change in the potassium concentration of the medium. The main compartments of six conical Warburg vessels each contained 0·5 ml. of a suspension of washed cells in water (9·5 mg. dry wt.) and 3·5 ml. of a solution consisting of 0·4 ml. of M-sodium acetate, 0·1 ml. of 0·2 M-sodium fumarate, 2 ml. of 0·2 M-potassium chloride and 32·5 ml. of the growth medium (without glucose) described earlier. The side arms of two vessels contained 0·5 ml. of water and those of the four others 0·5 ml. of ^{42}KCl solution. One of each set contained oxygen as gas phase and 2 N-NaOH solution in the centre well, the other nitrogen and yellow phosphorus.

Table 9. *Effect of sodium chloride on the distribution of potassium between cells and medium*

Each cup contained 0·5 ml. of cell suspension in water (17·6 mg. of dry cells); 1 ml. of 0·5 M-triethanolamine buffer, pH 7·4; 0·1 ml. of 0·23% KCl; 0·1 ml. of 0·1 M-sodium acetate; 0·1 ml. of 0·02 M-sodium fumarate; 0·2 ml. of 1% Difco yeast extract; 1·5 ml. of water in 1 and 2 and 1·5 ml. of 0·90% NaCl in 3 and 4. The contents of 1 and 3 were used for the determination of potassium, of 2 and 4 for the determination of sodium. Incubation for 60 min. at 15° in O_2.

Cup no. ...	1	2	3	4
	No NaCl		NaCl added	
Supernatant	42·1 μg. of K/ml.	116 μg. of Na/ml.	28·3 μg. of K/ml.	1490 μg. of Na/ml.
Cells	645 μg. of K/ml.	~0 μg. of Na/ml.	1325 μg. of K/ml.	80·2 μg. of Na/ml.

Table 10. *Simultaneous determination of changes in the concentrations of potassium, sodium and chloride in cells incubated in the presence of glutamate*

Cell suspension (4 ml., 58·4 mg. of dry cells); 2 ml. of 0·0287% KCl; 0·16 ml. of 0·25 M-sodium glutamate; 1·84 ml. of water. Incubation for 60 min. at 15°. Data 'before incubation' refer to cells suspended in 8 ml. of water (without KCl and glutamate); this suspension was analysed immediately after preparation. The data for the medium are given in order to indicate the magnitude of the concentration gradients between cells and medium.

			Medium		Cells	
Expt. no.	Gas phase	Ion	Before incubation (mM)	After incubation (mM)	Before incubation (mM)	After incubation (mM)
1	O_2	K	0·17	1·26	3·79	10·4
		Cl	~0	1·06	~0	~0
		Na	0·20	4·91	2·41	9·14
2	N_2	K	0·51	1·06	2·62	5·81
		Cl	~0	0·96	0·52	0·12
		Na	0·46	6·54	1·77	14·8

Table 11. *Distribution of* ^{42}K *between cells and medium*

For experimental conditions see text.

Cup no.	1	2	3	4	5	6
Gas phase	O_2	N_2	O_2	N_2	O_2	N_2
Period of incubation (min.)	0	0	5	5	30	30
Weight of residue after removal of supernatant (mg.)	66·8	62·7	64·0	60·5	70·8	67·9
Volume of residue (μl.)	65·6	61·6	62·9	59·4	69·6	66·7
Volume of cells in residue (μl.)	39·2	36·6	41·2	35·7	44·8	37·9
Volume of supernatant in residue (μl.)	26·4	25·0	21·7	23·7	24·8	28·8
K in supernatant (μg./ml.)	422	431	484	488	488	511
K in total residue (μg.)	93	64	89	64	99	67
K in total supernatant of residue (μg.)	9·9	9·6	10·5	11·5	12·1	14·7
K in total cells of residue (μg.)	83·1	54·4	78·5	52·5	86·9	52·3
K in 1 ml. of cells (μg.)	2120	1485	1 905	1 470	1 940	1 380
Ratio: $\frac{\text{K in cells}}{\text{K in medium}}$	4·78	3·45	3·93	3·01	3·96	2·69
Counts in supernatant (counts/min./ml.)	—	—	716 450	797 300	776 350	823 200
Specific activity of K in supernatant (counts/min./μg. of K)	—	—	1 480	1 635	1 590	1 610
Counts in total residue (counts/min.)	—	—	95 385	75 385	151 494	102 712
Counts in supernatant of residue (counts/min.)	—	—	15 546	18 896	19 253	23 708
Counts in total cells of residue (counts/min.)	—	—	79 840	56 490	132 240	79 005
Counts in 1 ml. of cells (counts/min.)	—	—	1 938 000	1 582 000	2 950 500	2 083 000
Specific activity of K in cells (counts/min./μg. of K)	—	—	1 015	1 075	1 520	1 510
Ratio: $\frac{\text{specific activity of K in cells}}{\text{specific activity of K in medium}}$	—	—	68·4	65·8	95·6	93·8
O_2 absorbed (μl./ml. of cells)	—	—	—	—	1 250	—

The cups were first shaken at 30° for 20 min. to establish a steady state between medium and cells. The first pair were then removed for the determination of the potassium values in cells and medium before the addition of ^{42}K. In the others ^{42}K was added from the side arm at this stage and shaking was continued for 5 and 30 min. respectively. The results are shown in Table 11. The concentration gradient between cells and medium was of the order of 3–4 in favour of the cells, and was consistently higher aerobically than anaerobically. Both aerobically and anaerobically extracellular potassium exchanged rapidly with the intracellular potassium. The exchange was slightly more rapid in oxygen than in nitrogen. After 5 min. the specific activity of the intracellular potassium was 68·4 % of the extracellular potassium in oxygen, and 65·8 % in nitrogen. After 30 min. the corresponding figures were 96·5 and 93·8 %. Thus almost all the intracellular potassium exchanged readily with the K^+ ions of the medium.

Comparison of different strains of Alcaligenes faecalis. The experiments so far discussed were all carried out with strain 1347. Some experiments were repeated with two other strains (19 and 415) but no major differences were found. Cells of these strains also lose most of their intracellular potassium on washing with water and recover it on suspension in a potassium-containing medium.

DISCUSSION

The need for potassium in bacterial growth (Rahn, 1935), the role of potassium as a cofactor in enzyme systems (Lardy & Ziegler, 1945; Lwoff & Ionescu, 1947; Nossal, 1951), the accelerating effect of potassium on bacterial respiration (Quastel & Webley, 1942) and the accumulation of potassium by bacterial cells (Cowie, Roberts & Roberts, 1949; Roberts, Roberts & Cowie, 1949; Carroll, Danby, Eddy & Hinshelwood, 1950; Eddy, Carroll, Danby & Hinshelwood, 1951) are all well-established phenomena. The present experiments reveal an organism in which the intracellular potassium is much less firmly bound than in organisms which have formerly been studied, in that most of the intracellular potassium is removed from the cells by washing with distilled water. The fact that the cells can recover potassium added to the suspension fluid in the absence of energy sources shows that the cells contain a material which binds potassium. The 'bound' potassium, as indicated by experiments with ^{42}K, readily exchanges with the K^+ ions of the medium. In this respect the potassium-binding material behaves like a colloid electrolyte or an ion-exchange resin.

Several authors have discussed the presence of potassium-binding cell constituents with reference to the uneven distribution of potassium between

cells and medium, but the previous search for potassium-binding substances has not proved successful (Roberts *et al.* 1949; Steinbach, 1950). The observations reported in this paper strongly support the view that *Alcaligenes faecalis* contains a potassium-binding constituent, but no statements can as yet be made about the nature of the potassium-binding reaction. Further work on disrupted cells aiming at the isolation and study of the potassium-binding material is required.

A potassium-binding material would account for the concentration gradients under anaerobic conditions and in the absence of oxidizable substrate. Superimposed upon that 'passive' transport of potassium is a mechanism of active transport which increases the concentration gradients in respiring cells.

SUMMARY

1. Cells of *Alcaligenes faecalis* lost most of their intracellular potassium when washed with distilled water. This treatment also removes most of the capacity to respire.

2. Respiration is restored by the addition of potassium (and an oxidizable substrate) to the suspending medium. The restoration is accompanied by an uptake of potassium against a concentration gradient.

3. The distribution of potassium between cells and medium was studied under a variety of conditions. Substantial amounts of potassium (up to 1·8 mg./ml. of cells) are accumulated in the cells against concentration gradients. Accumulation also occurs anaerobically in the absence of substrates but the final gradients reached are lower than in respiring cells.

4. Measurements of the simultaneous movements of potassium, sodium and chloride show that the uptake of potassium is not accompanied by an equivalent movement of chloride or release of sodium. How electroneutrality is maintained when potassium ions move has not been established.

5. The distribution of radioactivity between cells and medium after addition of ^{42}K to the medium shows that the intracellular potassium exchanges readily with the K^+ ions of the medium. The exchange was slightly more rapid aerobically than anaerobically.

6. It is concluded that the organism contains a potassium-binding substance which behaves similarly to a colloid electrolyte or an ion-exchange resin.

The work reported in this paper was supported by a grant from the Rockefeller Foundation.

REFERENCES

Carroll, T. C. N., Danby, C. J., Eddy, A. A. & Hinshelwood, C. N. (1950). *J. chem. Soc.* p. 946.
Conway, E. J. & O'Malley, E. (1946). *Biochem. J.* **40**, 59.
Cowie, D. B., Roberts, R. B. & Roberts, I. Z. (1949). *J. cell. comp. Physiol.* **34**, 243.
Domingo, W. R. & Klyne, W. (1949). *Biochem. J.* **45**, 400
Eddy, A. A., Carroll, T. C. N., Danby, C. J. & Hinshelwood, C. N. (1951). *Proc. Roy. Soc.* B, **138**, 219.
Lardy, H. A. & Ziegler, J. A. (1945). *J. biol. Chem.* **159**, 343.
Lwoff, A. & Ionescu, H. (1947). *C.R. Acad. Sci., Paris*, **225**, 77.
Nossal, P. M. (1951). *Biochem. J.* **49**, 407.
Quastel, J. H. & Webley, D. M. (1942). *Biochem. J.* **36**, 8.
Rahn, O. (1935). *J. Bact.* **32**, 393.
Roberts, R. B., Roberts, I. Z. & Cowie, D. B. (1949). *J. cell. comp. Physiol.* **34**, 259.
Sanderson, P. H. (1952). *Biochem. J.* **52**, 502.
Steinbach, H. B. (1950). *Amer. J. Physiol.* **163**, 236.

16α-Hydroxyoestrone in the Urine of Pregnant Women

By G. F. MARRIAN, K. H. LOKE, ELIZABETH J. D. WATSON and MARTA PANATTONI
Department of Biochemistry, University of Edinburgh

(*Received* 11 *September* 1956)

Watson & Marrian (1955) detected the presence in extracts of the urine of pregnant women of a ketonic Kober chromogen which was more 'polar' than oestrone, and, on solvent-partition evidence, concluded that it was 16-oxo-oestradiol-17β (16-oxo-oestra-1:3:5-triene-3:17β-diol). More recently Marrian, Watson & Panattoni (1957) concentrated this Kober chromogen (KC-5) and showed conclusively that the major component in it must have been either 16α-hydroxyoestrone (17-oxo-oestra-1:3:5-triene-3:16α-diol) or 16β-hydroxyoestrone (17-oxo-oestra-1:3:5-triene-3:16β-diol), and not 16-oxo-oestradiol-17β. On biogenetic grounds the view was favoured that the major component in KC-5 was 16α-hydroxyoestrone rather than 16β-hydroxyoestrone, but since reduction with sodium borohydride yielded a mixture of oestriol (oestra-1:3:5-triene-3:16α:17β-triol) and 16-*epi*oestriol (oestra-1:3:5-triene-3:16β:17β-triol) it was suggested that either 16β-hydroxyoestrone or arti-

factually produced 16-oxo-oestradiol-17β might also have been present in the isolated material.

For the further investigation of the nature of KC-5 it was clearly necessary to isolate a larger amount of more rigorously purified material from urine, and, since it seemed probable that the major component in the Kober chromogen might be 16α-hydroxyoestrone, it was also decided to attempt to prepare the latter by partial synthesis.

RESULTS

Preparation and properties of 16α-*hydroxyoestrone*

16α-Hydroxyoestrone diacetate, m.p. 172–174·5°, was prepared from oestrone by the method of Leeds, Fukushima & Gallagher (1954). These authors recorded m.p. 179–180° for their preparation. However, it would appear that the latter higher melting point is that of a different polymorphic modification of the same substance, since a sample of the preparation of Leeds *et al.* (1954) kindly supplied to us by Dr Gallagher was found to melt at 168·5–172°, and this melting point was not depressed after admixture with our own preparation. Moreover, Dr Gallagher has informed us that he has also observed a lower-melting modification of the substance.

After reduction of the diacetate by sodium borohydride, followed by alkaline hydrolysis, products were obtained which, as judged by partition-chromatographic analysis used in conjunction with the Kober reaction, appeared to contain about 81–85% of oestriol and about 15–19% of 16-*epi*oestriol. It seems unlikely that the formation of this small proportion of a 16-*epi*oestriol-like product could have been due to the presence in the starting material of 16β-hydroxyoestrone diacetate or of 16-oxo-oestradiol-17β diacetate, both of which could be expected to yield mainly 16-*epi*oestriol on reduction by sodium borohydride followed by hydrolysis, since about the same relative amounts of the two products were formed irrespective of the number of times that the starting material had been recrystallized (Table 1). The possibility that 16-*epi*oestriol was formed as a result of rearrangement of 16α-hydroxyoestrone diacetate to 16-oxo-oestradiol-17β diacetate during the reduction has not been excluded, but since the relative amounts of the two products could not be significantly altered by varying either the duration of the reaction or the amount of sodium borohydride used (Table 1) this explanation seems an unlikely one. A more probable explanation would seem to be that reduction of the 17-oxo group in 16α-hydroxyoestrone diacetate is less stereochemically specific for the formation of a 17β-hydroxyl group than it is in oestrone (cf. Biel, 1951), and that 17-*epi*oestriol (oestra-1:3:5-triene-3:16α:17α-triol) (Prelog, Ruzicka & Wieland, 1945), which is not clearly distinguishable from 16-*epi*oestriol in the solvent systems employed, was the 16-*epi*oestriol-like product which was formed.

Hydrolysis of 16α-hydroxyoestrone diacetate was effected somewhat as described by Leeds *et al.* (1954) for the rearrangement and hydrolysis of 16α:17α-epoxyandrostane-3β:17β-diol diacetate to 17-oxoandrostane-3β:16α-diol. After recrystallization the product melted at 238–240·5°, with marked shrinkage at about 216°, and had $[\alpha]_D^{16} + 180°$ (ethanol). Acetylation of the hydrolysis product

Table 1. *Reduction of* 16α-*hydroxyoestrone diacetate by sodium borohydride in methanolic solution: relative proportions of oestriol and* '16-epi*oestriol' in the products after alkaline hydrolysis*

Wt. of 16α-hydroxy-oestrone diacetate (mg.)	Previous recrystallizations	Wt. of $NaBH_4$ (mg.)	Reduction time (min.)	Composition of hydrolysed reduction product (%)	
				Oestriol	'16-*epi*-Oestriol'
20·3	2 × ethyl acetate–*n*-hexane	15 + 10	40 + 60	81·2	18·8
20·0	2 × ethyl acetate–*n*-hexane 1 × benzene–*n*-hexane 3 × methanol	15 + 10	40 + 60	81·5	18·5
21·0	2 × ethyl acetate–*n*-hexane 1 × benzene–*n*-hexane 3 × methanol	5 + 2	15 + 20	83·5	16·5
20·0	2 × ethyl acetate–*n*-hexane 2 × methanol	4 + 2·5	15 + 20	81·2	18·8
20·0	2 × ethyl acetate–*n*-hexane 2 × methanol	2·5	35	82·2	17·8
20·0	2 × ethyl acetate–*n*-hexane 1 × benzene–*n*-hexane 5 × methanol	4 + 2	15 + 20	85·3	14·7
5·3	2 × ethyl acetate–*n*-hexane 2 × methanol	5	40	81·8	18·2

yielded an acetate, m.p. 169–173°, $[\alpha]_D^{20}+149$ (ethanol), and on admixture with 16α-hydroxyoestrone diacetate the melting point was not depressed. It was evident, therefore, that the hydrolysis product of the diacetate was indeed 16α-hydroxyoestrone.

16α-Hydroxyoestrone was also prepared more conveniently by treatment of 16α:17α-epoxyoestra-1:3:5-triene-3:17β-diol diacetate with methanolic sulphuric acid at room temperature, when rearrangement and hydrolysis occurred (cf. Leeds *et al.* 1954).

The melting-point behaviour of 16α-hydroxyoestrone requires special comment. The melting point, like that of the crude KC-5 isolated by Marrian *et al.* (1957), was not depressed after admixture with 16-oxo-oestradiol-17β (m.p. 239–241·5°), and this fact suggested that at or near its melting point 16α-hydroxyoestrone might undergo rearrangement to 16-oxo-oestradiol-17β. On redetermining the melting point of 16α-hydroxyoestrone with a more rapid rate of heating over the range 205–230° it was seen that the shrinkage previously observed at about 216° was in fact a fairly sharp melt, followed immediately by resolidification which was complete at about 222°. This suggested that the lower melting point might be the true one of 16α-hydroxyoestrone and that the higher one might be that of the hypothetical rearrangement product. In fact 16α-hydroxyoestrone was found to yield, when heated at 220–230° *in vacuo*, 16-oxo-oestradiol-17β, which was identified by its physical properties and those of its diacetate.

Reduction of 16α-hydroxyoestrone by sodium borohydride yielded a mixture containing about 90 and 10% respectively of oestriol-like and 16-*epi*-oestriol-like products. Accordingly, as with the diacetate, it would appear either that some rearrangement occurred during the reaction or that a small proportion of 17-*epi*oestriol was formed. As catalytic reduction with hydrogen and platinum oxide in neutral ethanolic solution yielded a mixture which contained a somewhat higher proportion (30%) of the '16-*epi*oestriol-like' product than was found after sodium borohydride reduction, the latter explanation seems the more probable one, since under these conditions it is unlikely that any rearrangement of 16α-hydroxyoestrone to 16-oxo-oestradiol-17β would have occurred.

On reduction with sodium borohydride the KC-5 isolated from urine by Marrian *et al.* (1957) yielded a relatively high proportion (45%) of 16-*epi*-oestriol, which was identified as such with certainty; and in explanation of this finding the possibility was considered, among others, that the isolated material might have consisted of a mixture of 16α-hydroxyoestrone and 16-oxo-oestradiol-17β produced from the former by rearrangement during the extraction of the urinary phenolic fraction with aqueous sodium hydroxide. The fact that the urinary product had a significantly lower dextrorotation than that of 16α-hydroxyoestrone was clearly consistent with this possibility, and to investigate the latter further the behaviour of 16α-hydroxyoestrone in aqueous sodium hydroxide was studied.

In the preparation of the phenolic fraction of a urinary extract the time during which the fraction remains in alkaline solution is about 5–10 min.; accordingly, in the first instance, the effect on the optical rotation of 16α-hydroxyoestrone of allowing it to stand in aqueous N-sodium hydroxide for 10 min. at room temperature was examined. The phenolic product isolated from the reaction mixture showed a decrease in dextrorotation compared with that of the starting material which would have corresponded to the rearrangement of about 20% of the latter to 16-oxo-oestradiol-17β. That the observed decrease in dextrorotation was indeed due to partial rearrangement to 16-oxo-oestradiol-17β was shown by a second experiment in which 16α-hydroxyoestrone was treated with aqueous sodium hydroxide for 2 hr.: the product was identified as 16-oxo-oestradiol-17β by its physical properties and those of its diacetate. These findings support the view that the KC-5 isolated from pregnancy urine by Marrian *et al.* (1957) was a mixture of 16α-hydroxyoestrone and artifactually produced 16-oxo-oestradiol-17β.

Isolation, purification and identification of KC-5

By use of the procedures described by Marrian *et al.* (1957) crude KC-5, $[\alpha]_D^{18}+114°$ (ethanol), was obtained from enzymically hydrolysed late-pregnancy urine. An attempt to purify directly the dextrorotatory component in this product by recrystallization from methanol failed, and accordingly it was decided to attempt purification via the acetate.

The acetylated product yielded two crops of apparently pure crystalline material: (*a*) m.p. 172·5–174·5°, $[\alpha]_D^{20}+146°$ (ethanol); and (*b*) m.p. 170–173°, $[\alpha]_D^{18}+157°$ (ethanol). Analysis of (*a*) gave C and H values in good agreement with those required for a compound of the formula $C_{22}H_{26}O_5$.

Conclusive proof of the identity of KC-5 acetate and 16α-hydroxyoestrone diacetate was provided as follows: (i) The melting point of product (*b*) was not depressed after admixture with 16α-hydroxyoestrone diacetate. (ii) The absorption spectra of solutions of KC-5 acetate (*b*) and 16α-hydroxyoestrone diacetate in concentrated sulphuric acid over the range 220–550 mμ. after 2 hr. at 25° were identical (Fig. 1). (iii) The infrared spectra of KC-5 acetate (*b*) and 16α-hydroxyoestrone diacetate, which were determined in potassium chloride disks by Dr R. K. Callow of the National Institute

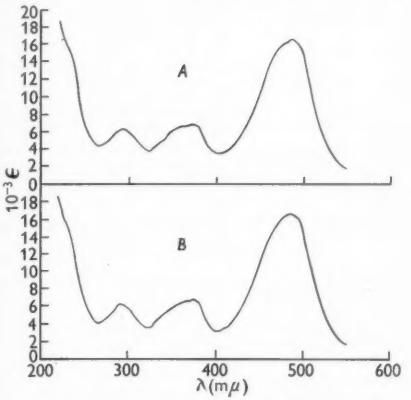


Fig. 1. Absorption spectra of H_2SO_4 solutions of (*A*) 16α-hydroxyoestrone diacetate and (*B*) KC-5 acetate after 2 hr. at 25°.

for Medical Research, 'showed complete similarity in all features, both in the main bands...and in all the bands in the "fingerprint" region...'.

Hydrolysis of KC-5 acetate with methanolic sulphuric acid at room temperature yielded a product which had $[\alpha]_D^{18}+169°$ (ethanol) and m.p. about 215° and 237·5–240°. The absorption spectrum of a solution of the product in concentrated sulphuric acid determined after 2 hr. closely resembled, but was not identical with, that of a similarly prepared solution of 16α-hydroxyoestrone. The slight differences between the absorption spectra and the fact that the optical rotation of the product was slightly low suggested that during the hydrolysis of KC-5 diacetate slight rearrangement had occurred. Unfortunately there was insufficient of the hydrolysis product to permit of more rigorous purification.

It is considered that completely satisfactory proof for the identity of KC-5 and 16α-hydroxyoestrone has been obtained.

EXPERIMENTAL

Methods

The procedures used for partition-chromatographic analysis, for the Kober reaction and for the determination of melting points, were as described by Marrian *et al.* (1957). All melting points are uncorrected for emergent stem.

Preparation and properties of 16α-*hydroxyoestrone*

16α-*Hydroxyoestrone diacetate.* This was prepared from 16α:17α-epoxyoestra-1:3:5-triene-3:17β-diol diacetate by treatment with perchloric and acetic acids as described by Leeds *et al.* (1954). The crude product of rearrangement was purified by filtration in benzene solution through a column of deactivated [10% (w/v) of water] Al_2O_3 and then by two crystallizations from ethyl acetate–*n*-hexane. The material had m.p. 169–172·5°, and partition-chromatographic analysis in the system 90% (v/v) ethanol in water/*n*-hexane did not reveal the presence of more than one Kober chromogen. Different portions of this material were further recrystallized from methanol and benzene–*n*-hexane. The portion for analysis was further recrystallized once from benzene–*n*-hexane and thrice from methanol, when it had m.p. 172–174·5°, $[\alpha]_D^{18}+126°$ (*c*, 0·502 in chloroform), $[\alpha]_D^{18}+153°$ (*c*, 0·503 in ethanol) (Found: C, 71·2; H, 6·8. Calc. for $C_{22}H_{26}O_5$: C, 71·3; H, 7·1%).

Reduction of 16α-*hydroxyoestrone diacetate by sodium borohydride.* The reduction of 16α-hydroxyoestrone diacetate by Na borohydride was studied in a series of experiments in which samples of the diacetate which had been recrystallized in different ways were allowed to react at room temperature in methanolic solution with varying amounts of the reductant for varying times. After hot alkaline hydrolysis the reaction mixtures were diluted with water, acidified with HCl and extracted with ether. The ether extracts were washed with 8·5% (w/v) $NaHCO_3$ and with water and evaporated to dryness. About 50 μg. of each product was analysed by partition chromatography, with the system 70% (v/v) methanol in water–ethylene dichloride, and in each case the presence of two different Kober chromogens chromatographically indistinguishable from oestriol and 16-*epi*oestriol respectively was detected. The more 'polar' Kober chromogen was isolated in one experiment and identified as oestriol by a mixed m.p. with an authentic specimen of the latter; in another experiment it was identified after acetylation by a mixed m.p. with authentic oestriol triacetate. Owing to lack of material conclusive evidence concerning the nature of the less 'polar' Kober chromogen was not obtained. The results of these experiments are summarized in Table 1.

Hydrolysis of 16α-*hydroxyoestrone diacetate.* To a solution of 170 mg. of 16α-hydroxyoestrone diacetate [m.p. 172–174·5°; $[\alpha]_D^{18}+150°$ (ethanol)] in 20 ml. of methanol was added 5 ml. of 5N-H_2SO_4 and the mixture allowed to stand at room temperature for 5 days. After dilution with 140 ml. of ethyl acetate the solution was washed with 8·5% (w/v) $NaHCO_3$ and water, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The product (130 mg.) on crystallization from methanol yielded 52 mg. of colourless crystals, m.p. about 216° and 238–240·5°, $[\alpha]_D^{16}+180°$ (*c*, 0·490 in ethanol) (Found: C, 75·6; H, 7·3. Calc. for $C_{18}H_{22}O_3$: C, 75·5; H, 7·7%).

Acetylation of a portion of this material with acetic anhydride and pyridine at room temperature overnight yielded a product which after crystallization from methanol had m.p. 169–173° and $[\alpha]_D^{20}+149°$ (*c*, 0·504 in ethanol). The melting point was not depressed after admixture either with the above-described 16α-hydroxyoestrone diacetate (m.p. 172–174·5°) or a sample of the latter (m.p. 168·5–172°) supplied by Dr T. F. Gallagher.

16α-*Hydroxyoestrone from* 16α:17α-*epoxyoestra*-1:3:5-*triene*-3:17β-*diol diacetate.* The epoxyacetate (45 mg.) was dissolved in a mixture of 4 ml. of methanol and 1 ml. of 5N-H_2SO_4, and the solution allowed to stand at room temperature for 5 days. After dilution with ethyl acetate the solution was washed with 85% (w/v) $NaHCO_3$ and water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The product (37 mg.) yielded 11 mg. of colourless needles after crystallization from methanol. These had m.p.

237–239° (shrinkage at 215° not recorded as a melt) and $[\alpha]_D^{17}$ +174° (*c*, 0·500 in ethanol). Acetylation at room temperature with acetic anhydride and pyridine yielded a product which had m.p. 167–172° after one crystallization from methanol. The melting point was not depressed after admixture with 16α-hydroxyoestrone diacetate (m.p. 172–174·5°).

Reduction of 16α-*hydroxyoestrone by sodium borohydride.* To a solution of 5·0 mg. of 16α-hydroxyoestrone in 2·5 ml. of methanol was added 5 mg. of Na borohydride. After standing at room temperature for 40 min. the solution was diluted with water, acidified with HCl and extracted with ether. The ether extract, after washing with $NaHCO_3$ and with water, was evaporated to dryness. Analysis by partition chromatography [70% (v/v) methanol in water–ethylene dichloride] of 50 μg. of the product showed the presence of 90 and 10% respectively of 'oestriol-like' and '16-*epi*oestriol-like' Kober chromogens.

Catalytic reduction of 16α-*hydroxyoestrone.* A solution of 155 μg. of 16α-hydroxyoestrone in 4 ml. of ethanol was shaken for 30 min. at atmospheric pressure with about 0·4 mg. of Adams platinum oxide catalyst in an atmosphere of hydrogen. After filtration the solution was evaporated to dryness, and a Girard separation with trimethylammonium-acetohydrazide chloride carried out on a portion of the product to remove any unreduced ketonic material. Analysis of the non-ketonic fraction by partition chromatography [70% (v/v) methanol in water–ethylene dichloride] showed the presence of 70 and 30% respectively of 'oestriol-like' and '16-*epi*oestriol-like' Kober chromogens.

Rearrangement of 16α-*hydroxyoestrone to* 16-*oxo-oestradiol-*17β *by heat.* 16α-Hydroxyoestrone ($[\alpha]_D^{18}$ +170° (ethanol); 25 mg.) was introduced into a tube, which was then sealed under reduced pressure ($1{\cdot}4 \times 10^{-4}$ mm. Hg). The tube was immersed in an oil bath which was rapidly heated to 220°, maintained at this temperature for 5 min., and finally heated to 230° for a further 2 min. After cooling, the slightly yellow semicrystalline product was washed several times with *n*-hexane and then crystallized once from methanol. The crystallized product melted at 236–238° with only very slight shrinkage at 217°, and had $[\alpha]_D^{18}$ −69° (*c*, 0·508 in ethanol). Acetylation at room temperature with acetic anhydride and pyridine yielded a product with m.p. 133–137°, and the m.p. after admixture with authentic 16-oxo-oestradiol-17β diacetate (m.p. 134–137°) was 133–136·5°.

Rearrangement of 16α-*hydroxyoestrone to* 16-*oxo-oestradiol-*17β *in alkaline aqueous solution.* To a solution of 10·4 mg. of 16α-hydroxyoestrone ($[\alpha]_D^{17}$ +181° (ethanol)) in 1 ml. ethanol was added 100 ml. of N-NaOH. After standing for 10 min. at room temperature sufficient 5N-H_2SO_4 was added to neutralize nine-tenths of the NaOH and neutralization (to pH 9) then completed by passing in a stream of CO_2. The reaction mixture was extracted with ether and the ether extract, after washing with 2% (w/v) $NaHCO_3$ and water and drying over anhydrous Na_2SO_4, was evaporated to dryness. The unpurified product showed $[\alpha]_D^{17}$ +122° (*c*, 0·50 in ethanol).

To investigate the possibility that the fall in optical rotation observed in this experiment had been due to the rearrangement of 16α-hydroxyoestrone to 16-oxo-oestradiol-17β, the following further experiment was carried out: To a solution of 48 mg. of 16α-hydroxyoestrone ($[\alpha]_D^{17}$ +181° (ethanol)) in 2 ml. of ethanol was added 100 ml. of N-NaOH. The solution was allowed to stand at room temperature for 2 hr. and then acidified with 5N-H_2SO_4. The reaction mixture was extracted with ether, and the extract, after washing with 5% (w/v) $NaHCO_3$ and water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product (39·5 mg.) had $[\alpha]_D^{20}$ −83° (*c*, 0·493 in ethanol). After crystallization once from methanol 19 mg. of material having m.p. 236·5–238·5° and $[\alpha]_D^{19}$ −95° (*c*, 0·492 in ethanol) was obtained. Acetylation of this in the usual way yielded a product which, after crystallization twice from ethyl acetate–*n*-hexane, had m.p. 133–136°, and the m.p. after admixture with authentic 16-oxo-oestradiol-17β diacetate was 133–137°.

Identification of KC-5 from pregnancy urine as 16α-*hydroxyoestrone*

Isolation of crude KC-5. By exactly the same methods as previously described by Marrian *et al.* (1957) a fraction weighing 125 mg. and containing 94 mg. of KC-5 (calc. as oestriol) was obtained by partition chromatography from the ether-soluble ketonic-phenolic fraction of 256 l. of enzymically-hydrolysed pooled late-pregnancy urine. Pigmented and gummy impurities were removed from this material by two washings with small volumes of methanol at −20°, and thus were obtained 64 mg. of a nearly white solid having $[\alpha]_D^{18}$ +114° (*c*, 0·495 in ethanol). After two crystallizations from methanol 5·9 mg. of material having $[\alpha]_D^{20}$ −41° (*c*, 0·523 in ethanol) were obtained. In view of this failure to concentrate the dextrorotatory component in the mixture directly all the fractions containing KC-5 were recombined, and then washed once with a small volume of methanol at −20° to remove pigment and gum.

Isolation of 16α-*hydroxyoestrone diacetate from acetylated crude KC-5.* The crude KC-5 (63 mg.) obtained as described above was treated with 2 ml. of acetic anhydride and 2 ml. of pyridine overnight at room temperature. One crystallization from ethyl acetate–*n*-hexane and three from methanol yielded 15·7 mg. of a product (*a*) which had m.p. 172·5–174·5° and $[\alpha]_D^{20}$ +146° (*c*, 0·529 in ethanol) (Found: C, 71·3; H, 6·9. Calc. for $C_{22}H_{26}O_5$: C, 71·3; H, 7·1%).

From the material obtained after combining the mother liquors of the crystallizations described above a further batch (*b*) of 18·7 mg. of material was obtained by washing once with a small volume of methanol at −20° and crystallizing once from ethyl acetate–*n*-hexane. This had m.p. 170–173° and $[\alpha]_D^{18}$ +157° (*c*, 0·512 in ethanol). The mixed m.p. with authentic 16α-hydroxyoestrone diacetate was 170–173°.

Hydrolysis of KC-5 acetate. To a solution of 19·6 mg. of a mixture of batches (*a*) and (*b*) of the purified acetate in 4 ml. of methanol was added 0·5 ml. of 5N-H_2SO_4 and the mixture allowed to stand at room temperature for 5 days. After dilution with ethyl acetate the reaction mixture was washed with 8·5% (w/v) $NaHCO_3$ and water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The product was crystallized once from a small volume of methanol at −20°, yielding 11·5 mg. of material having m.p. about 215° and 237·5–240° and $[\alpha]_D^{18}$ +169° (*c*, 0·501 in ethanol). The mixed m.p. with authentic 16α-hydroxyoestrone (m.p. about 216° and 238–240·5°) was about 217° and 238–240·5°.

DISCUSSION

The results reported here have conclusively shown the presence of 16α-hydroxyoestrone in extracts of enzymically hydrolysed late-pregnancy urine: and

in view of the finding that this compound readily undergoes rearrangement in alkaline solution to 16-oxo-oestradiol-17β there can be little doubt that the KC-5 previously isolated by Marrian *et al.* (1957) was indeed a mixture of 16α-hydroxyoestrone and artifactually produced 16-oxo-oestradiol-17β as was suggested. The available evidence strongly suggests that extracts of late-pregnancy urine contain no 16-oxo-oestradiol-17β other than that which must be artifactually formed from 16α-hydroxyoestrone during the extraction of the phenolic fraction by aqueous alkali from ether. In view of this the recent claim by Levitz, Spitzer & Twombly (1956) to have detected the presence of radioactive 16-oxo-oestradiol-17β in the urine of human subjects after the administration of [16-^{14}C]oestradiol-17β must be treated with reserve.

The isolation of 16α-hydroxyoestrone from urine lends support to the previous suggestion of Marrian *et al.* (1957) that this compound may be formed from oestrone by 16α-hydroxylation and may be the metabolic intermediate in the 'hydration' of oestrone to oestriol.

SUMMARY

1. 16α-Hydroxyoestrone diacetate, prepared from 16α:17α-epoxyoestra-1:3:5-triene-3:17β-diol diacetate by the method of Leeds *et al.* (1954), yielded 16α-hydroxyoestrone on cold acid hydrolysis. 16α-Hydroxyoestrone was also prepared directly from the epoxyacetate by cold acid treatment.

2. 16α-Hydroxyoestrone readily undergoes rearrangement to 16-oxo-oestradiol-17β in aqueous alkali at room temperature. The same rearrangement occurs when it is heated *in vacuo* to 220–230°.

3. A preparation of the ketonic-phenolic Kober chromogen (KC-5) prepared from enzymically-hydrolysed late-pregnancy urine as described by Marrian *et al.* (1957) yielded a product after acetylation which has been conclusively identified as 16α-hydroxyoestrone diacetate.

4. In view of the rearrangement undergone by 16α-hydroxyoestrone in alkaline solution it is concluded that KC-5 as isolated from urine by the methods described consists of 16α-hydroxyoestrone contaminated with about 20% of artifactually produced 16-oxo-oestradiol-17β.

5. It is suggested that 16α-hydroxyoestrone may be the metabolic intermediate between oestrone and oestriol.

The authors are grateful to the Medical Research Council for a grant from which the expenses of this work were defrayed. They are also grateful to their colleague Dr J. W. Minnis, who carried out all the microanalyses; to Dr T. E. Gallagher of the Sloan-Kettering Institute, New York, who generously provided a sample of authentic 16α-hydroxyoestrone diacetate; to Dr R. K. Callow of the National Institute for Medical Research, who very kindly determined the infrared spectra; and to their colleagues in the Department of Obstetrics and Gynaecology and in the Clinical Endocrinology Research Unit (M.R.C.) who so willingly provided facilities for the collection of urine specimens.

One of the authors (K. H. L.) is indebted to the Federation of Malaya for the award of a Queen's Scholarship.

REFERENCES

Biel, J. H. (1951). *J. Amer. chem. Soc.* **73**, 847.
Leeds, N. S., Fukushima, D. K. & Gallagher, T. F. (1954). *J. Amer. chem. Soc.* **76**, 2943.
Levitz, M., Spitzer, J. R. & Twombly, G. H. (1956). *Fed. Proc.* **15**, 299.
Marrian, G. F., Watson, E. J. D. & Panattoni, M. (1957). *Biochem. J.* **65**, 12.
Prelog, V., Ruzicka, L. & Wieland, P. (1945). *Helv. chim. Acta*, **28**, 250.
Watson, E. J. D. & Marrian, G. F. (1955). *Biochem. J.* **61**, xxiv.

Synthesis of Porphyrins by Cell Suspensions of *Tetrahymena vorax*: Effect of Members of the Vitamin B Group

By JUNE LASCELLES*
Microbiology Unit, Department of Biochemistry, University of Oxford

(*Received* 13 *September* 1956)

It is well established that the biosynthesis of the porphyrin nucleus in animal tissues proceeds by condensation of glycine with an unsymmetrical derivative of succinate (derived from the tricarboxylic acid cycle) with intermediate formation of δ-aminolaevulic acid (ALA) and porphobilinogen (Shemin, 1955; Dresel & Falk, 1956*a–c*; Falk, Dresel, Benson & Knight, 1956; Berlin, Neuberger & Scott, 1956*a*, *b*). There is evidence that a similar pathway occurs in photosynthetic bacteria of the Athiorhodaceae group (Lascelles, 1955; Cooper, 1956).

* Member of Scientific Staff, Medical Research Council.

Less is known of the individual enzymes catalysing these various steps. Gibson, Neuberger & Scott (1955) have studied the properties of purified ALA dehydrase, which converts ALA into porphobilinogen. This enzyme is widely distributed in higher animals and bacteria; it has also been found in preparations from spinach leaves, *Chlorella* and *Tetrahymena* (Granick, 1954). Enzyme fractions from spinach and from chick erythrocytes, which convert porphobilinogen into unidentified precursors of porphyrins, have also been briefly reported (Bogorad, 1955; Granick, 1955). The enzymes responsible for the initial reaction between glycine and the succinate derivative have not been isolated and the mechanism of the reaction is obscure.

Studies of animal nutrition have shown that many B group vitamins are concerned, directly or indirectly, in haemopoiesis (see Lemberg & Legge, 1949; Jukes, 1953). In particular, dietary deficiency of pyridoxine results in hypochromic anaemia in pigs, dogs and rats (Chick, Macrae, Martin & Martin, 1938; Fouts, Helmer & Lepkovsky, 1940; Kornberg, Tabor & Sebrell, 1945). The concentration of free protoporphyrin in the erythrocytes of pyridoxine-deficient pigs was found by Cartwright & Wintrobe (1948) to be markedly lower than that in the control animals, suggesting that this vitamin is directly concerned in the synthesis of protoporphyrin. Hypochromic anaemia has also been observed in pantothenate-deficient rats (Carter, Macfarlane, O'Brien & Robb-Smith, 1945).

The experiments with growing animals do not show the step or steps in the synthesis of porphyrin at which the vitamins act. This was the aim of the present work, with micro-organisms as experimental material, and an organism was sought which required for growth a number of B group vitamins and which also formed free porphyrins in easily detectable quantities. The photosynthetic bacteria were not suitable since they require only a limited number of B group vitamins for growth, and the free-living ciliate *Tetrahymena vorax* was finally chosen. The nutrition of this group of organisms has been studied in great detail and growth can be obtained in a completely defined medium (Kidder & Dewey, 1951; Kidder, 1953). Furthermore, free protoporphyrin is formed by cultures of some strains of *Tetrahymena* (Rudzinska & Granick, 1953); the strain used in the present work was particularly active in forming porphyrins during growth under certain conditions (personal communication from Dr V. Dewey). Porphyrin synthesis from glycine and from ALA was studied with washed organisms harvested from media containing growth-limiting concentrations of B group vitamins; respiratory activity was also examined, as a check of the possible indirect effects arising from the vitamin deficiency.

EXPERIMENTAL

Organism and media

Tetrahymena vorax, strain V 2, was obtained from Dr V. Dewey. Stock cultures were maintained in 2% (w/v) peptone (Evans Medical Supplies Ltd., Liverpool) adjusted to pH 7·0. These were subcultured fortnightly and incubated for 48 hr. at 30°; they were stored at room temperature (about 20°).

The organisms for experiments with suspensions were harvested from the semi-defined medium T, which contained per litre: acid-hydrolysed casein (vitamin-free), prepared by the method of Snell & Rannefeld (1945), equivalent to 2·5 g. of original casein; DL-tryptophan, 140 mg.; L-cysteine hydrochloride, 100 mg.; glucose, 3·6 g.; $Na_2HPO_4,12H_2O$, 1·4 g.; KH_2PO_4, 540 mg.; $MgSO_4,7H_2O$, 100 mg.; $MnSO_4,4H_2O$, 1 mg.; adenine, guanine and uracil, 10 mg. each; Tween 80 (Honeywill and Stein Ltd., London, S.W. 1), 0·4 ml. The complete medium contained the following concentrations of B group vitamins: nicotinic acid, 8 μM; thiamine hydrochloride, 3 μM; calcium pantothenate, 4·2 μM in terms of pantothenic acid; riboflavin, 1·3 μM; pyridoxal hydrochloride, 0·4 μM; pteroylglutamic acid, 0·1 μM; DL-6-thioctic acid, 25 $\mu\mu$M; biotin, 4 $\mu\mu$M. The pH was adjusted to 7·4 with N-NaOH. With the exception of pyridoxal, all additions were made before autoclaving for 10 min. at 10 lb./in.^{2} pressure.

Growth of organisms

Organisms were grown in Roux bottles containing 100 ml. of medium T modified as indicated. Each bottle was sown with 0·5 ml. (about 0·25 mg. dry wt. of cells) of a 48 hr. culture grown at 30° in 2% (w/v) Evans peptone; the bottles were incubated at 37° for 48 hr. The growth attained (about 0·5 mg. dry wt./ml. of culture) varied from 50 to 70% of that reached on more prolonged incubation (72 hr.) under the same conditions. It was assessed in an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with a neutral density filter.

Procedures for study of porphyrin synthesis

Harvesting of organisms. The cultures were centrifuged gently at 500 *g* for 4 min., the supernatant fluid was removed quickly by suction and the cells were washed as above in half the original culture volume of 0·85% NaCl. They were finally suspended in the salts solution described below. The dry weight was determined by heating samples to constant weight at 90–100° and correcting the values for the weight of added salts.

Synthesis from glycine. The washed organisms were suspended in a mixture of salts similar to that used by Ryley (1952); it contained: NaCl, 0·047 M; KCl, 0·002 M; $MgSO_4$, 0·001 M; $MnSO_4$, 5×10^{-6} M; phosphate buffer, pH 7·3, 0·025 M (prepared from 0·2 M-Na_2HPO_4 and -KH_2PO_4). The other components of the reaction mixture (I) were: glycine, 0·02 M; sodium fumarate, 0·02 M; pyridoxal hydrochloride, 10^{-6} M; Tween 80, 0·1%; penicillin G and streptomycin sulphate, each 20 μg./ml. The final volume was 2·5 ml. and the concentration of organisms 3–4 mg. dry wt./ml. Incubation was in 18 mm. × 150 mm. tubes for 48 hr. at 37°; the tubes were held in a sloped position to increase aeration. The antibiotics were added to prevent growth of bacteria during incubation of the suspensions; they had no observed effect on the metabolism of *T. vorax*.

Synthesis from δ-aminolaevulic acid. The organisms were incubated in a mixture of inorganic salts similar to that described above, with addition of penicillin G and streptomycin sulphate, each 20 μg./ml., and mM-δ-aminolaevulic acid hydrochloride (mixture II). The conditions of incubation were as for studying synthesis from glycine.

Estimation and identification of porphyrins

Estimation. Most of the porphyrin formed by the organisms passed out into the suspending fluid, but a small proportion (5–10%) was retained by the cells. Samples of the whole reaction mixture (cells and suspending fluid) were diluted in 1·4N-HCl, thereby removing the free porphyrin present in, or absorbed on to, the cells. The samples were centrifuged and the optical density of the supernatant fluid was measured at the Soret peak (405–407 mμ.); the Unicam spectrophotometer SP. 600 (Unicam Instruments Ltd., Cambridge) was used. Since uroporphyrin was the major component of the mixtures of porphyrins formed, the concentration of porphyrin was calculated in terms of this compound, the extinction coefficient of Rimington & Sveinsson (1950) being used.

The proportion of uro-, copro- and proto-porphyrin was determined by the fractionation procedure described previously (Lascelles, 1956*a*).

Identification. The porphyrins formed were identified by the paper-chromatographic methods of Eriksen (1953), Chu, Green & Chu (1951) and Falk & Benson (1953); samples were prepared for chromatography by the methods described before (Lascelles, 1956*a*).

Manometric experiments

The conventional Warburg technique was used. Each vessel contained cells (3–4 mg. dry wt./ml.) suspended in the salts mixture described above, together with glucose (0·02M); the final volume was 2·5 ml. The centre well of each vessel contained 20% NaOH and a pleated filter paper. Incubation was in air at 37°. The results are expressed as Q_{O_2} values (μl. of oxygen consumed/mg. dry wt. of organisms/hr.). The Q_{O_2} values for cells grown on the complete medium T varied from 20 to 30 with different batches of cells.

Chemicals

Details concerning the solutions of ALA, iron citrate and of the porphyrins used as reference standards for chromatography have been given previously (Lascelles, 1956*a*).

RESULTS

Porphyrin formation during growth

Porphyrins were formed by growing cultures of *T. vorax* in medium T; addition of glycine (0·01M) to the medium, which contained acid-hydrolysed casein, increased the formation of porphyrin. Glucose was essential for production of porphyrin though the organism could grow on medium T without it.

The organism grows well at 30°, but appreciable formation of porphyrin was observed only in cultures incubated at 37°; Dr V. Dewey (private communication) made similar observations with cultures grown in a peptone medium. All further experiments were carried out with suspensions of organisms grown in medium T at 37°.

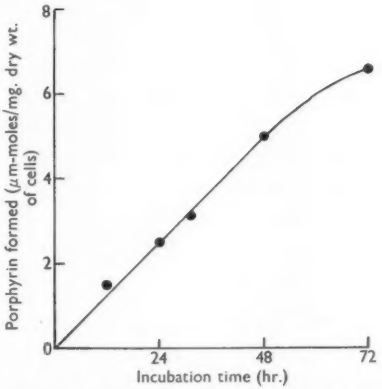


Fig. 1. Rate of porphyrin synthesis by cell suspensions. The washed organisms (3·3 mg. dry wt./ml.) were harvested from medium T and were incubated for varying periods in mixture I at 37°.

Table 1. *Effect of glucose during growth on porphyrin synthesis by harvested organisms*

Organisms were grown for 48 hr. in medium T with various amounts of glucose. Harvested cells were incubated for 48 hr. in mixture I or II.

Concn. of glucose in growth medium (M)	Porphyrin formed (μm-moles/mg. dry wt. of organisms)	
	Mixture I	Mixture II
0	0	5·2
0·005	1·3	5·2
0·01	4·2	5·6
0·02*	5·0	5·8

* Normal concn. in medium T.

Conditions for synthesis by suspensions of organisms

When the washed organisms were incubated aerobically in mixture I at 37° porphyrin synthesis occurred at a rate which was linear for 48 hr. or more (Fig. 1). The rate of synthesis was directly proportional to the concentration of organisms within the range 1·5–4 mg. dry wt./ml. When the suspensions were incubated at 30° the amount of porphyrin formed was less than 20% of that formed at 37°. No porphyrins were formed when the suspensions were incubated anaerobically in mixture I.

Organisms harvested after growth in medium T without glucose or with low concentrations of the sugar formed little or no porphyrin from glycine (Table 1); addition of glucose to such suspensions

incubated in mixture I did not restore activity. Omission of glycine from mixture I reduced synthesis to about 40 % of that in the complete mixture (Table 2). Omission of fumarate had a less-marked effect, and other carboxylic acids tested were less active or inactive (Table 2). Acetate inhibited porphyrin synthesis both in the presence and absence of fumarate. Tween 80 was also included in mixture I, as it increased synthesis slightly; it is necessary for growth of this organism in medium T.

δ-Aminolaevulic acid promoted porphyrin synthesis in the absence of both glycine and fumarate (Table 2). Further experiments with this substance were in mixture II, which contained neither Tween 80 nor pyridoxal; these compounds did not affect porphyrin synthesis from ALA. Organisms grown without glucose formed porphyrin from ALA though not from glycine (Table 1).

Effect of B group vitamins

T. vorax requires for growth nicotinic acid, thiamine, riboflavin, pantothenic acid, pteroylglutamic acid, thioctic acid and pyridoxal or pyridoxamine; its requirements for B group vitamins are therefore similar to those of *Tetrahymena geleii* (Kidder & Dewey, 1951; Kidder, 1953). With the exception of pyridoxal, addition of these vitamins, either singly or as mixtures, did not increase porphyrin synthesis by suspensions of organisms harvested from medium T; pyridoxal sometimes increased porphyrin formation from glycine (see later) and it was therefore normally included in mixture I.

The effect of the B group vitamins was examined in organisms rendered deficient in the vitamin in question by growing them with suboptimum concentrations. Usually the concentration was sufficient to give between 60 and 80 % of the growth attained on the complete medium T.

Pyridoxal. Organisms grown with suboptimum concentrations of pyridoxal formed little porphyrin from glycine. The Q_{O_2} value of the deficient cells was similar to that of the control cells, whereas the amount of porphyrin formed was less than 20 % of that synthesized by the normal organisms (Table 3).

Table 2. *Substrates required for porphyrin synthesis by suspensions of organisms*

Organisms harvested from medium T were incubated for 48 hr. in mixture I without glycine and fumarate, with the additions shown. The carboxylic acids were added as their sodium salts.

Additions			Porphyrin formed (μm-moles/mg. dry wt. of organisms)
Glycine (M)	Fumarate (M)	Other compounds	
0	0	—	0·8
0	0·02	—	1·9
0·005	0·02	—	4·1
0·01	0·02	—	4·4
0·02	0·02	—	5·0
0·04	0·02	—	4·9
0·02	0	—	3·9
0·02	0·005	—	4·4
0·02	0·01	—	5·0
0·02	0	Succinate (0·02 M)	4·6
0·02	0	Acetate (0·02 M)	2·8
0·02	0	α-Oxoglutarate (0·02 M)	3·5
0	0	ALA (0·001 M)	4·2

Table 3. *Effect of deficiency of pyridoxal on porphyrin synthesis*

Organisms were harvested from medium T containing various concentrations of pyridoxal. They were incubated for 48 hr. in mixture I with or without pyridoxal (10^{-6} M), or in mixture II.

Expt. no.	Concn. of pyridoxal in growth medium (M)	Growth* (%)	Q_{O_2}	Porphyrin formed (μm-moles/mg. dry wt. of organisms)		
				Mixture I		
				Without pyridoxal	With pyridoxal	Mixture II
1	10^{-8}	71	20	0·7	2·5	4·3
	2×10^{-8}	81	24	0·6	2·1	4·3
	4×10^{-7} (normal concn.)	—	25	4·8	5·0	4·4
2	$2{\cdot}5 \times 10^{-8}$	85	23	1·0	5·6	—
	4×10^{-7}	—	24	4·2	5·8	—

* Growth attained by the deficient culture as % of that reached in the control culture.

Table 4. *Effect of deficiency of pantothenate on porphyrin synthesis*

Deficient organisms were grown on medium T containing 8×10^{-8}M-pantothenate, and normal organisms on the same medium with 4×10^{-6}M-pantothenate; the growth of the deficient cultures was 68–75% of that in the controls. The harvested organisms were incubated for 48 hr. either in mixture I supplemented with pantothenate (4×10^{-5}M) where shown, or in mixture II.

Expt. no.	Type of organisms	Q_{O_2}	Porphyrin formed (μm-moles/mg. dry wt. of organisms)		
			Mixture I		
			Unsupplemented	Supplemented	Mixture II
1	Deficient	26	2·0	1·9	3·4
	Normal	22	4·3	4·2	2·7
2	Deficient	22	1·5	1·5	5·1
	Normal	24	4·4	—	4·9
3	Deficient	22	1·2	1·1	4·0
	Normal	23	3·8	—	3·8

Table 5. *Effect of deficiency of riboflavin on porphyrin synthesis*

Organisms were harvested from medium T containing varying concentrations of riboflavin. The suspensions were incubated for 48 hr. in mixture I or in mixture II, supplemented with riboflavin (2×10^{-6}M) where indicated.

Expt. no.	Concn. of riboflavin in growth medium (M)	Growth* (%)	Q_{O_2}	Porphyrin formed (μm-moles/mg. dry wt. of organisms)			
				Mixture I		Mixture II	
				Unsupplemented	Supplemented	Unsupplemented	Supplemented
1	2×10^{-8}	60	28	1·5	2·9	2·1	2·6
	4×10^{-8}	70	29	2·9	4·1	2·7	3·4
	10^{-6} (normal concn.)	—	29	5·0	4·8	6·5	6·5
2	2×10^{-8}	70	29	2·3	5·1	2·0	3·2
	10^{-6}	—	25	5·8	5·8	4·3	4·4

* Growth attained by the deficient culture as percentage of that reached in the control culture.

Addition of pyridoxal to suspensions of the deficient organisms increased their activity; in some experiments pyridoxal also increased porphyrin formation by cells harvested from media containing optimum concentrations of the vitamin (Table 3). Pyridoxal phosphate also increased synthesis by the deficient cells but it had only about one-half of the activity of pyridoxal on a molar basis. The phosphorylated vitamin is also less active for growth of *Tetrahymena* (Kidder & Dewey, 1951).

Porphyrin synthesis from ALA was not affected by deficiency of pyridoxal (Table 3, last column).

Pantothenate. Deficiency of pantothenate during growth reduced the ability of the organisms to synthesize porphyrin from glycine but did not affect synthesis from ALA (Table 4). The effect was observed only with organisms grown with concentrations of pantothenate which limited growth to 75% or less of the normal value. The Q_{O_2} values for such cells did not differ significantly from those of normal cells, whereas the amount of porphyrin formed from glycine varied from 30–50% of that synthesized by the controls. Addition of pantothenate to suspensions of the deficient cells did not increase porphyrin synthesis (Table 4).

Table 6. *Effect of deficiency of nicotinic acid, thiamine, thioctic acid and pteroylglutamic acid on porphyrin synthesis*

In each experiment organisms were grown in medium T containing the modified concentrations of the vitamins shown in parentheses. The washed organisms were incubated for 48 hr. in mixture I. Results are expressed as % of the values obtained with normal organisms harvested from medium T.

Cells deficient in	Growth of culture*	Activity compared with normal organisms (%)	
		Q_{O_2}	Porphyrin synthesis
Nicotinic acid (2×10^{-7}M)	68	100	52
Thiamine (3×10^{-9}M)	95	83	40
Thioctic acid (5×10^{-10}M)	60	95	66
Pteroylglutamic acid ($2{\cdot}5 \times 10^{-9}$M)	70	100	95

* Growth of culture from which the deficient organisms were harvested as percentage of that reached by the control culture.

Riboflavin. Of the growth factors concerned in the hydrogen-transport mechanisms of the cell, riboflavin had the most marked influence on porphyrin synthesis (Table 5). Organisms grown with limiting concentrations of this vitamin synthesized only 30–50 % of the amount of porphyrin formed by normal cells, though the Q_{O_2} values did not differ. Synthesis from both glycine and from ALA was decreased in organisms deficient in riboflavin; in both cases addition of the vitamin partially restored synthesis (Table 5).

Other vitamins. Organisms deficient in nicotinic acid, thiamine and thioctic acid also had reduced activity with glycine (Table 6), but synthesis from ALA was not impaired. Deficiency in pteroylglutamic acid did not significantly affect formation of porphyrin (Table 6).

Effect of iron

Previous work with the photosynthetic organism *Rhodopseudomonas spheroides* showed that iron salts markedly influenced porphyrin formation (Lascelles, 1956*a*). Addition of iron to suspensions of this organism almost completely prevented porphyrin synthesis from glycine plus α-oxoglutarate. Synthesis from ALA was not prevented by iron but in the presence of iron both protoporphyrin and haematin were formed (Lascelles, 1956*a*, *b*). These substances were not formed in the absence of iron.

Addition of iron citrate to suspensions of *T. vorax* decreased, but did not abolish, porphyrin synthesis from glycine and from ALA (Table 7). However, cells harvested from medium T containing added iron formed no detectable porphyrin from glycine; porphyrin was formed from ALA by the iron-rich cells but the yield was decreased (Table 7). The proportion of protoporphyrin formed by such cells was increased (see later).

Types of porphyrin formed

The porphyrins formed by the suspensions were examined by paper chromatography and by extraction with organic solvents followed by fractionation with HCl (see Methods). Cells harvested from medium T formed a mixture of porphyrins of identical composition when incubated in either mixture I or II. Uroporphyrin predominated, accounting for 50–60 % of the total porphyrins (Fig. 2, Expt. *A*); it behaved mainly as the type III isomer when subjected to paper chromatography by the method of Falk & Benson (1953). Protoporphyrin was also formed (20–30 % of the total) together with coproporphyrin III (15–25 % of the total). These three porphyrins are also formed predominantly by chick-erythrocyte preparations when incubated with ALA or with porphobilinogen (Falk *et al.* 1956).

Effect of iron and pantothenate. Organisms harvested from medium T supplemented with iron citrate also formed a mixture of uro-, proto- and copro-porphyrins from ALA, but the proportion of protoporphyrin was increased at the expense of uroporphyrin; there was little change in the relative amount of coproporphyrin (Fig. 2, Expt. *B*).

Deficiency of pantothenate did not affect the ability of the cells to form uro- and copro-porphyrin from ALA (see above), but synthesis of protoporphyrin was decreased (Fig. 2, Expt. *C*). The effect of deficiency of pantothenate on the formation of protoporphyrin from ALA was more pronounced in organisms grown in the presence of iron

Table 7. *Effect of iron on porphyrin synthesis*

Organisms were grown on medium T without added iron ('normal organisms') or on the same medium supplemented with 4×10^{-6} M-iron citrate ('iron-rich organisms'). The harvested organisms were incubated for 48 hr. in mixture I or II with addition of iron citrate as shown.

Type of organisms	Iron citrate concn. (M)	Porphyrin formed (μm-moles/mg. dry wt. of organisms) Mixture I	Mixture II
Normal	0	4·7	4·9
	5×10^{-6}	4·9	4·0
	10^{-5}	2·7	3·7
'Iron-rich'	0	0	3·0

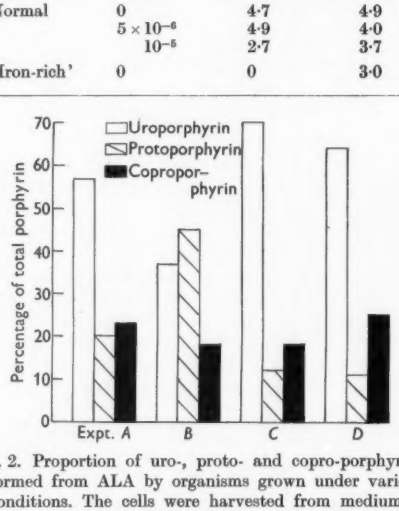


Fig. 2. Proportion of uro-, proto- and copro-porphyrins formed from ALA by organisms grown under various conditions. The cells were harvested from medium T (Expt. *A*), medium T supplemented with 4×10^{-6} M-iron citrate (Expt. *B*), from medium T containing a decreased concentration (8×10^{-8} M) of pantothenate (Expt. *C*), and from medium T containing 8×10^{-8} M-pantothenate and supplemented with 4 μM-iron citrate (Expt. *D*). In each experiment the washed cells were suspended in mixture II and distributed in ten replicate tubes (2·5 ml./tube); incubation was for 48 hr. The replicates were pooled and the proportion of the various porphyrins was determined (see Experimental).

salts (Fig. 2, Expt. *D*). Of the total porphyrin formed by such organisms, protoporphyrin accounted for about 11 % and uroporphyrin for about 64 %; the protoporphyrin formed by organisms grown with optimum concentrations of pantothenate, together with added iron, accounted for 45 % of the total porphyrin (Fig. 2, Expt. B).

DISCUSSION

The accumulation of free porphyrins by this strain of *T. vorax* probably occurs as a result of some derangement at one or more stages in the synthesis of intracellular haem compounds such as cytochromes and haemoglobin; these pigments have been detected in the cells of *T. geleii* (Ryley, 1952; Keilin & Ryley, 1953). The free porphyrins may not be true intermediates in the synthesis of the haem compounds but may be stabilized by-products arising from such intermediates. Dresel & Falk (1956*c*), using isotopic techniques, have provided evidence that free uro-, copro- and proto-porphyrins are not intermediates in the conversion of glycine, ALA or porphobilinogen into haem in erythrocyte preparations from chickens, though they are undoubtedly closely related to the genuine intermediates.

The results suggest that the path of porphyrin synthesis in *T. vorax* is similar to that occurring in higher animals. Glycine increased synthesis by the washed organisms, and ALA was converted into porphyrins. Addition of possible sources of the 4-C intermediate required for condensation with glycine had little effect on synthesis; the intermediate may have been provided by oxidation of endogenous material. The amount of oxidizable substances within the organisms was apparently large, since addition of substrates such as glucose did not increase the Q_{O_2} values; similar findings have been made with suspensions of *T. geleii* (Ryley, 1952). The 4-C intermediate may have arisen from the tricarboxylic acid cycle, individual reactions of which have been demonstrated in *T. geleii* (Seaman, 1955).

The experiments with cells deficient in vitamin B_6 indicate that this factor (presumably in coenzyme form) is necessary for conversion of glycine into porphyrins; synthesis from ALA was not affected. The effect of pyridoxal could be shown even with cells harvested from media containing concentrations of the vitamin sufficient to support full growth. Schulman & Richert (1955) have briefly reported that deficiency of vitamin B_6 in the diet reduces the incorporation of ^{14}C-labelled glycine and succinate into haem by preparations of duck erythrocytes; activity is restored by pyridoxal phosphate. The condensation between glycine and the 4-C intermediate in porphyrin synthesis may be similar to that between glycine and a 1-C derivative to give serine; this latter reaction is mediated by pyridoxal phosphate in both bacteria and in animal tissues (Lascelles & Woods, 1954; Blakley, 1955).

The participation of pantothenate in the initial condensation reaction is also suggested by the experiments with deficient organisms. A similar finding has been made with haemolysates from pantothenate-deficient ducks, which incorporate labelled glycine and succinate into haem less actively than do preparations from normal birds (Schulman & Richert, 1956). The vitamin may be needed for the formation of succinyl-coenzyme A, possibly the 4-C derivative required for condensation with glycine. This compound is a product of the oxidative decarboxylation of α-oxoglutarate in both animal tissues and in some bacteria (Gunsalus, 1953; Sanadi & Littlefield, 1953). There is also evidence that it is formed by the same reaction in cell-free extracts of *T. geleii* (Seaman, 1953). Pantothenate may also be concerned in the formation of protoporphyrin from ALA. Cells deficient in pantothenate showed decreased synthesis of protoporphyrin from ALA with a corresponding increase in the amount of uroporphyrin, suggesting that coenzyme A may participate in the oxidative decarboxylation necessary to produce the vinyl side chains of protoporphyrin.

The effects of nicotinic acid, thiamine and thioctic acid on porphyrin synthesis from glycine could be atrributed to their participation in the tricarboxylic acid cycle, in particular at the stage of oxidative decarboxylation of α-oxoglutarate (Gunsalus, 1953). The need for thioctic acid in the oxidation of α-oxoglutarate by extracts of *T. geleii* has been shown by Seaman (1953); the keto acid also accumulates in cultures of this organism grown with suboptimum concentrations of thioctic acid (Dewey & Kidder, 1952). Deficiency of riboflavin decreased porphyrin synthesis both from glycine and from ALA, suggesting that the vitamin may act in the oxidation of porphobilinogen (formed from ALA) to porphyrin; the overall reaction requires the loss of 6 atoms of hydrogen and 4 molecules of ammonia.

The inability of cells grown with added iron salts to form porphyrins from glycine is consistent with observations made with photosynthetic bacteria (Lascelles, 1955). Another effect of iron, observed with both photosynthetic bacteria and *Tetrahymena*, is in the conversion of ALA into protoporphyrin; with both types of organism iron salts increase the formation of this porphyrin, whereas the proportion of uroporphyrin or coproporphyrin is decreased. Thus this metal has similar effects on porphyrin synthesis by organisms containing bacteriochlorophyll as their main pyrrole component (together with smaller quantities of haematin

compounds) and by those containing only haematin compounds. The mode and locus of action of iron as well as of the B group vitamins cannot be finally ascertained until the enzymes involved in the formation of porphyrins from glycine have been separated and resolved.

SUMMARY

1. Suspensions of *Tetrahymena vorax* synthesize porphyrins when incubated aerobically at 37° with glycine and fumarate; they also convert δ-aminolaevulic acid into porphyrins.

2. Synthesis from glycine by organisms harvested from media containing growth-limiting concentrations of pyridoxal or pantothenate is reduced to 30% or less of that achieved by cells grown with optimum concentrations of these vitamins.

3. Cells deficient in riboflavin, nicotinic acid, thiamine or thioctic acid also form less porphyrin from glycine than do normal cells.

4. Synthesis from δ-aminolaevulic acid by cells deficient in riboflavin is reduced; deficiency in the other vitamins does not decrease the amount of porphyrins formed from this substrate.

5. The porphyrins formed from either glycine or δ-aminolaevulic acid consist of a mixture of uroporphyrin III, protoporphyrin and coproporphyrin III. Cells deficient in pantothenate form less protoporphyrin than do normal cells.

6. Organisms harvested from media containing added iron salts do not form porphyrins from glycine, but do so from δ-aminolaevulic acid. Such cells form more protoporphyrin from this substrate than do organisms grown without added iron.

7. It is concluded that pyridoxal and pantothenate may be concerned directly in the primary stage of porphyrin biosynthesis, which involves condensation between glycine and a 4-C intermediate.

I am indebted to Professor D. D. Woods, F.R.S., for his patient advice and encouragement.

REFERENCES

Berlin, N. I., Neuberger, A. & Scott, J. J. (1956*a*). *Biochem. J.* **64**, 80.

Berlin, N. I., Neuberger, A. & Scott, J. J. (1956*b*). *Biochem. J.* **64**, 90.

Blakley, R. L. (1955). *Biochem. J.* **61**, 315.

Bogorad, L. (1955). *Science*, **121**, 878.

Carter, C. W., Macfarlane, R. G., O'Brien, J. R. P. & Robb-Smith, A. H. T. (1945). *Biochem. J.* **39**, 339.

Cartwright, G. E. & Wintrobe, M. M. (1948). *J. biol. Chem.* **172**, 557.

Chick, H., Macrae, T. F., Martin, A. J. P. & Martin, C. J. (1938). *Biochem. J.* **32**, 2207.

Chu, T. C., Green, A. A. & Chu, E. J. (1951). *J. biol. Chem.* **190**, 643.

Cooper, R. (1956). *Biochem. J.* **63**, 25P.

Dewey, V. C. & Kidder, G. W. (1952). *Proc. Soc. exp. Biol., N.Y.*, **80**, 302.

Dresel, E. I. B. & Falk, J. E. (1956*a*). *Biochem. J.* **63**, 72.

Dresel, E. I. B. & Falk, J. E. (1956*b*). *Biochem. J.* **63**, 80.

Dresel, E. I. B. & Falk, J. E. (1956*c*). *Biochem. J.* **63**, 388.

Eriksen, L. (1953). *Scand. J. clin. Lab. Invest.* **5**, 155.

Falk, J. E. & Benson, A. (1953). *Biochem. J.* **55**, 101.

Falk, J. E., Dresel, E. I. B., Benson, A. & Knight, B. C. (1956). *Biochem. J.* **63**, 87.

Fouts, P. J., Helmer, O. M. & Lepkovsky, S. (1940). *Amer. J. med. Sci.* **199**, 163.

Gibson, K. D., Neuberger, A. & Scott, J. J. (1955). *Biochem. J.* **61**, 618.

Granick, S. (1954). *Science*, **120**, 1105.

Granick, S. (1955). *Abstr. Amer. chem. Soc.* p. 69C. 128th Meeting, Minneapolis.

Gunsalus, I. C. (1953). In *The Mechanism of Enzyme Action*, p. 545. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.

Jukes, T. H. (1953). In *Biochemistry and Physiology of Nutrition*, vol. 1, p. 328. Ed. by Bourne, G. H. & Kidder, G. W. New York: Academic Press Inc.

Keilin, D. & Ryley, J. F. (1953). *Nature, Lond.*, **172**, 451.

Kidder, G. W. (1953). In *Symposium on Nutrition and Growth Factors, 6th Int. Congr. Microbiol., Rome*, p. 44.

Kidder, G. W. & Dewey, V. C. (1951). In *Biochemistry and Physiology of Protozoa*, vol. 1, p. 323. Ed. by Lwoff, A. New York: Academic Press Inc.

Kornberg, A., Tabor, H. & Sebrell, W. H. (1945). *Amer. J. Physiol.* **143**, 434.

Lascelles, J. (1955). In *The Biosynthesis of Porphyrins and Porphyrin Metabolism, Ciba Foundation Conf.* p. 265. Ed. by Wolstenholme, G. E. W. London: J. and A. Churchill Ltd.

Lascelles, J. (1956*a*). *Biochem. J.* **62**, 78.

Lascelles, J. (1956*b*). *J. gen. Microbiol.* **15**, 404.

Lascelles, J. & Woods, D. D. (1954). *Biochem. J.* **58**, 486.

Lemberg, R. & Legge, J. W. (1949). *Haematin Compounds and Bile Pigments.* London: Interscience Publ. Ltd.

Rimington, C. & Sveinsson, S. L. (1950). *Scand. J. clin. Lab. Invest.* **2**, 209.

Rudzinska, M. A. & Granick, S. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 525.

Ryley, J. F. (1952). *Biochem. J.* **52**, 483.

Sanadi, D. R. & Littlefield, J. W. (1953). *J. biol. Chem.* **201**, 103.

Schulman, M. P. & Richert, D. A. (1955). *J. Amer. chem. Soc.* **77**, 6402.

Schulman, M. P. & Richert, D. A. (1956). *Fed. Proc.* **15**, 349.

Seaman, G. R. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 184.

Seaman, G. R. (1955). In *Biochemistry and Physiology of Protozoa*, vol. 2, p. 91. Ed. by Hutner, S. H. & Lwoff, A. New York: Academic Press Inc.

Shemin, D. (1955). In *The Biosynthesis of Porphyrins and Porphyrin Metabolism, Ciba Foundation Conf.* p. 4. Ed. by Wolstenholme, G. E. W. London: J. and A. Churchill Ltd.

Snell, E. E. & Rannefeld, A. N. (1945). *J. biol. Chem.* **157**, 475.

The Biochemistry of Aromatic Amines

3. ENZYMIC HYDROXYLATION BY RAT-LIVER MICROSOMES*

BY J. BOOTH AND E. BOYLAND
Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W. 3

(*Received* 8 *October* 1956)

Several hydroxylating systems showing different intracellular distributions but requiring a reduced phosphopyridine nucleotide, or the nucleotide and an enzyme system capable of reducing it, have been described. Mueller & Miller (1948) reported that a rat-liver homogenate, fortified with diphosphopyridine nucleotide (DPN^+), nicotinamide, magnesium ions and hexose diphosphate, demethylates and hydroxylates 4-dimethylaminoazobenzene to give 4′-hydroxy derivatives. Kensler, Miller & Hyatt (1956) showed that the hydroxylation could be carried out by rat-liver microsomes in the presence of the soluble liver fraction. The system responsible for the 11β-hydroxylation of 11-deoxycorticosterone by suspensions of ox-adrenocortical mitochondria requires reduced triphosphopyridine nucleotide (TPNH) (Hayano & Dorfman, 1954; Grant & Brownie, 1955); the 21-hydroxylation of 17α-hydroxyprogesterone requires the same coenzyme, but for this reaction to take place both the microsomes and the soluble fraction are needed (Ryan & Engel, 1956). Aniline and acetanilide are hydroxylated, chiefly in the *p*-position, by rabbit-liver microsomes and this sytem requires TPNH and oxygen (Brodie *et al.* 1955; Mitoma & Udenfriend, 1955; Mitoma, Posner, Reitz & Udenfriend, 1956), whereas the conversion of 2-acetamidofluorene into the 7-hydroxy derivative by rat-liver homogenate takes place in the presence of DPN^+, nicotinamide and succinate (Peters & Gutman, 1956).

Two examples of naturally occurring compounds which are hydroxylated by similar systems have been reported, but in these the microsomes are not involved. A soluble enzyme system from rat liver which catalyses the conversion of phenylalanine into tyrosine requires reduced diphosphopyridine nucleotide (DPNH) and oxygen (Udenfriend & Cooper, 1952; Mitoma, 1956), and the conversion of kynurenine into 3-hydroxykynurenine by liver mitochondria of cats and rats requires TPNH (de Castro, Price & Brown, 1956).

The isolation of derivatives of 2-amino-1-naphthol and 2-amino-6-naphthol from the urine of animals dosed with 2-naphthylamine and 2-acetamidonaphthalene has demonstrated that hydroxylation of the amines occurs *in vivo* (Wiley, 1938; Dobriner, Hofmann & Rhoads, 1941; Manson & Young, 1950; Boyland & Manson, 1955) and *in vitro* experiments have shown that the reaction also occurs in the presence of rat-liver slices (Booth, Boyland & Manson, 1955). The present paper reports an investigation on the enzyme system concerned with this reaction.

* Part 2: Boyland, Manson & Orr (1957).

EXPERIMENTAL

Materials. Triphosphopyridine nucleotide (TPN^+) as the dihydrate of the sodium salt, TPNH as the sodium salt, glucose 6-phosphate (G 6-P) as the sodium salt or the heptahydrate of the barium salt and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co. Potassium 2-naphthyl sulphamate and potassium 6-hydroxy-2-naphthyl sulphamate were prepared by the action of chlorosulphonic acid on 2-naphthylamine and 2-amino-6-naphthol respectively [Boyland *et al.* 1957; Boyland & Manson (in preparation)]. Other derivatives of 2-naphthylamine were obtained as described in a previous paper (Booth *et al.* 1955). Pyrophosphate buffers were prepared from solutions of $Na_2H_2P_2O_7$ and $K_4P_2O_7$.

Liver preparations. In experiments to determine the intracellular location of the enzyme, male rat-liver homogenates in 0·25 M-sucrose were fractionated by the method of Schneider (1948) and the sedimented material was resuspended in 0·2 M-pyrophosphate buffer, pH 7·2.

To obtain an active 'microsome preparation', chilled livers of male rats were homogenized with an MSE Ato-Mix Blender (Measuring and Scientific Equipment Co. Ltd.) for 0·5 min. or with a Potter & Elvehjem (1936) homogenizer in 4 vol. of 0·05 M-pyrophosphate buffer, pH 7·2. The homogenates were centrifuged for 10 min. at 600 *g* to remove cell debris and nuclei and the supernatant was centrifuged for 10 min. at 22 000 *g* in a Spinco Model L preparative ultracentrifuge with rotor no. 40, since this force gave maximum activity in the supernatant under these conditions. The supernatant (100 ml.) was brought to pH 5·4 by the addition of 10% (v/v) acetic acid (approximately 6 ml.), allowed to stand for 0·5 hr. and centrifuged at 10 500 *g* for 30 min. The supernatant was discarded, and the precipitate, consisting of microsomes and some liver dehydrogenases, was suspended in 100 ml. of pyrophosphate buffer, pH 7·2, and dialysed against 5 l. of the same buffer solution for approximately 18 hr. All operations were carried out between 0° and 8° and the centrifugal forces refer to the centre of the tube employed. The preparations correspond to 200 mg. of

liver/ml. and the protein content, determined by a modification of the biuret method (Fincham, 1954), was 9 mg. of protein/ml.

Estimation of hydroxylating activity with 2-acetamidonaphthalene as substrate. The activity was determined by estimating the rate of formation of 2-acetamido-6-naphthol from 2-acetamidonaphthalene. Incubations were carried out in air in flasks which were shaken for 30 min. at 38°. The standard reaction mixture consisted of 3 mM-G 6-P, 0·2 mM-TPN, 35 mM-nicotinamide, 2 mM-2-acetamidonaphthalene and 1 ml. of microsome preparation in a total volume of 3 ml. of pyrophosphate buffer (0·2 M), pH 7·2. The 2-acetamidonaphthalene was added in ethylene glycol monomethyl ether (0·1 ml.), and the reaction was stopped by the addition of 10% (w/v) Na_2CO_3 (1 ml.). The reaction mixture was poured into a glass-stoppered tube, the flask washed out with *n*-butanol (3 ml.) and the washings were added to the tube. The addition of diazotized sulphanilic acid (0·5 ml.), freshly prepared by the addition of 0·5% $NaNO_2$ (1·6 ml.) to 0·2% sulphanilic acid in 0·1 N-HCl (10 ml.), followed by shaking and centrifuging to separate the layers, produced an orange colour in the *n*-butanol phase; this phase (2·5 ml.) was removed to a 5 ml. graduated tube containing 2 N-NaOH (2 ml.). After shaking and allowing the layers to separate, the *n*-butanol layer was yellow owing to the presence of 2-naphthylamine which had been formed by deacetylation of the substrate, and the red colour produced by the 2-acetamido-6-naphthol was in the alkaline layer. This was removed (2 ml.) and added to another tube containing ethanol (0·5 ml.) to prevent turbidity, and the optical density was read at 520 mμ on a Unicam SP. 500 spectrophotometer. A standard curve was prepared by the estimation of known amounts of 2-acetamido-6-naphthol added to a tissue preparation under the same conditions and the relationship between optical-density readings and μmoles of compound added was linear up to 0·4 μmole. Flasks in which the tissue preparation had been boiled were run as blanks in each series of experiments.

In experiments to determine the intracellular distribution of the enzyme it was necessary to precipitate the protein by heating in a water bath at 70° for 2 min. After centrifuging, the precipitated protein was shaken with *n*-butanol (3 ml.) which was then added to the supernatant and the colour developed as before.

Identification of hydroxylated metabolites of various substrates. The microsome preparation was incubated for 2 hr. in the reaction mixture described for the quantitative experiments, the various substrates being added in ethylene glycol monomethyl ether (0·1 ml.). The addition of the less-soluble substrates resulted in some of them being precipitated, and in these experiments they remained partly in suspension throughout the incubation. With 2-naphthylamine it was not possible to detect 2-amino-1-naphthol, but when a phosphate buffer fortified with adenosine triphosphate (ATP), $MgCl_2$ and K_2SO_4 to facilitate sulphate conjugation (Bernstein & McGilvery, 1952) was employed, 2-amino-1-naphthylsulphuric acid could be identified.

Identification of metabolites by paper chromatography. At the end of the incubation period the reaction mixture was poured into a glass-stoppered tube and shaken with ethyl acetate (1 ml.). After centrifuging to separate the layers the ethyl acetate phase was applied to Whatman no. 1 chromatography paper and descending chromatograms were run for approximately 18 hr. in the solvent system *n*-butanol–*n*-propanol–water (2:1:1, by vol.). Authentic specimens of the hydroxy compounds were run alongside the metabolites in each case. The following sprays were employed for identification by colour reactions: (*a*) hexylresorcinol–N-HCl followed by $NaNO_2$ (0·5%) and then by hexylresorcinol (0·5% in 2 N-NaOH); (*b*) 2:6-dichloroquinonechloroimide (0·5% in ethanol) followed by Na_2CO_3 (10%); (*c*) 1-naphthol–N-HCl followed by $NaNO_2$ (0·5%) and then by 1-naphthol (0·5% in 2 N-NaOH); (*d*) diazotized sulphanilic acid [1·6 ml. of $NaNO_2$ (0·5%) added to 10 ml. of sulphanilic acid (0·2% in N-HCl)] followed by Na_2CO_3 (10%); (*e*) NNCD reagent (saturated 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate in 0·1 N-HCl) followed by Na_2CO_3 (10%); (*f*) *p*-dimethylaminobenzaldehyde (0·5% in ethanol containing 1 ml. of conc. HCl/100 ml.); (*g*) diazotized *p*-nitroaniline [1·6 ml. of $NaNO_2$ (0·5%) added to 10 ml. of *p*-nitroaniline (0·2% in N-HCl)] followed by Na_2CO_3 (10%).

Identification of metabolites by colour tests on the reaction mixture. (*a*) 2-Acetamido-7-hydroxyfluorene and 2-amino-7-hydroxyfluorene were identified by the 'nitrite test' before and after hydrolysis with N-HCl for 1 hr. in a boiling-water bath (Damron & Dyer, 1953). (*b*) Phenol was identified by adding a saturated solution of NNCD reagent in 0·1 N-HCl (1 ml.), 5 N-NaOH (0·5 ml.) and *n*-butanol (3 ml.) to the reaction mixture and shaking. The red colour produced, both by the reaction mixture and by an authentic specimen of phenol, was extracted into the *n*-butanol phase and had a maximum absorption at 530 mμ. The formation of phenol from benzene was confirmed with 2:6-dichloroquinonechloroimide. The reaction mixture was shaken with *n*-butanol (3 ml.) and centrifuged. After breaking the emulsion with a glass rod and re-centrifuging, the *n*-butanol phase was removed. After the addition of 0·5% 2:6-dichloroquinonechloroimide in ethanol (0·1 ml.) and 0·1 M-borate (2 ml.) the tube was allowed to stand for 0·5 hr. with occasional shaking. The blue colour in the *n*-butanol produced by the reaction mixture and by an authentic specimen of phenol had an absorption maximum at 640 mμ. A blank in which the tissue preparation has been boiled was run at the same time in both cases. (*c*) *p*-Acetamidophenol was detected by the indophenol reaction essentially as described by Brodie & Axelrod (1948).

RESULTS

Distribution and requirements of the enzyme system

When the rat-liver homogenate was fractionated according to the method of Schneider (1948) it was found that the hydroxylating activity was present in the microsome fraction (Table 1). Equal activity was obtained in the presence of either chemically prepared TPNH, TPN^+ and the soluble liver fraction or TPN, G 6-P and G 6-P dehydrogenase. In the microsome preparations used in the quantitative experiments both the microsomes and the dehydrogenases of the liver are present, only the material soluble at pH 5·4 having been discarded. Therefore with this preparation no activity was obtained unless a substrate for one of the dehydrogenases such as G 6-P, glucose or to a less extent malate, was also added. The replacement of TPNH

Table 1. *Intracellular distribution and requirements of enzyme system responsible for the conversion of 2-acetamidonaphthalene into 2-acetamido-6-naphthol*

Flasks contain 2 mM-2-acetamidonaphthalene in a total volume of 3 ml. of pyrophosphate buffer (0·2M), pH 7·2.

Liver preparation (1 ml.)	Phosphopyridine nucleotide (0·2 mM)	Substrate for dehydrogenase (3 mM)	G 6-P dehydrogenase (0·1%)	Gas	2-Acetamido-6-naphthol (μmoles/g. of liver/hr.)
Whole homogenate	TPN+	–	–	Air	1·0
Nuclei	TPN+	–	–	Air	0
Nuclei	TPNH	–	–	Air	0
Mitochondria	TPN+	–	–	Air	0
Mitochondria	TPNH	–	–	Air	0
Microsomes	TPN+	–	–	Air	0
Soluble fraction	TPN+	–	–	Air	0
Microsomes + soluble fraction	TPN+	–	–	Air	1·1
Microsomes + soluble fraction	TPN+	–	–	N_2	0
Microsomes	TPNH	–	–	Air	1·2
Microsomes	TPN+	G 6-P	+	Air	1·3
Microsomes	DPNH	–	–	Air	0·6
Microsome preparation	TPN+	–	–	Air	0
Microsome preparation	TPN+	G 6-P	–	Air	1·2
Microsome preparation	TPN+	G 6-P	+	Air	1·2
Microsome preparation	TPN+	Glucose	–	Air	1·0
Microsome preparation	TPN+	Malate	–	Air	0·3
Microsome preparation	TPNH	–	–	Air	1·1
Microsome preparation	DPNH	–	–	Air	0·5

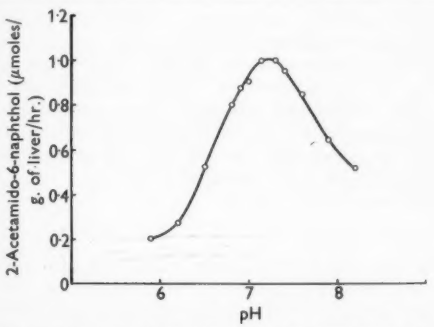


Fig. 1. Effect of pH on the rate of hydroxylation of 2-acetamidonaphthalene. Reaction mixtures (3 ml.) contained 1 ml. of microsome preparation, 3 mM-G 6-P, 0·2 mM-TPN, 35 mM-nicotinamide, 2 mM-2-acetamidonaphthalene and 0·2M-pyrophosphate buffer, and were incubated for 30 min. at 38°.

by DPNH resulted in a loss of approximately half of the activity and complete loss of activity resulted when air was replaced by nitrogen.

Formation of 2-acetamido-6-naphthol from 2-acetamidonaphthalene

Rate of reaction and effect of pH. The rate of reaction was constant over 30 min. and was directly proportional to the volume of microsome preparation added between 0·2 and 1 ml. The estimation of activity at various pH values in the range 5·8–8·2 and with 1 ml. of microsome preparation incubated for 30 min., showed optimum activity at pH 7·2, the rate of formation of 2-acetamido-6-naphthol being 1 μmole/g. of liver/hr. (Fig. 1). The activity of the microsome preparation was dependent on either TPNH or a TPNH-generating system such as TPN^+, G 6-P and G 6-P dehydrogenase. Sufficient liver-G 6-P dehydrogenase was precipitated by the treatment with acetic acid, since the microsome preparation showed the same activity as a suspension of microsomes in the soluble liver fraction, and addition of G 6-P or G 6-P dehydrogenase did not increase the activity or affect the pH–activity curve.

Effect of concentrations of the reactants. The rate of hydroxylation was determined in a series of experiments in which the concentration of one of the reactants was varied while the others were present in concentrations giving maximum activity. The effect of variations in TPN concentration is shown in Fig. 2, a half-maximal rate being obtained with a concentration of 0·018 mM-TPN.

The results of experiments in which the activity was measured in the presence of various concentrations of G 6-P (Fig. 3) show a half-maximal rate with 0·25 mM-G 6-P.

When the substrate concentration was varied, maximal activity occurred at a concentration of 2 mM; at higher substrate concentrations the reaction rate was diminished (Fig. 3). Examination by paper chromatography showed that some of the substrate was deacetylated to 2-naphthylamine and this compound may have inhibited the oxidation. However, estimations of 2-naphthylamine at the end of the incubations showed that the concentration was 0·07 mM, whereas a concentration of

0·25 mM-2-naphthylamine caused only 32% inhibition, suggesting that it was the substrate itself which caused a decrease in activity at concentrations above 2 mM.

Hydroxylation of other aromatic compounds

The hydroxylated metabolites identified after treating other aromatic compounds with the microsome preparation in the presence of TPN^+, nicotinamide and G 6-P are listed in Table 2. These experiments were purely qualitative and the conditions which produce maximum hydroxylation of 2-acetamidonaphthalene were employed.

When the three acetylated amines acetanilide, 2-acetamidonaphthalene and 2-acetamidofluorene were used as substrates, deacetylation occurred in each case, the free amine being identified on the paper chromatograms. No hydroxy derivatives of the deacetylated products, however, were identified except 2-amino-7-hydroxyfluorene from 2-acetamidofluorene, but it is uncertain whether the deacetylation or the hydroxylation occurs first. No acetylated hydroxy compounds were found in experiments in which the free amines were used as substrates.

The hydroxy compounds formed from the acetylated amines acetanilide, 2-acetamidonaphthalene and 2-acetamidofluorene were *p*-acetamidophenol, 2-acetamido-6-naphthol and 2-acetamido-7-hydroxyfluorene respectively, the hydroxylation having taken place at the carbon atom which is furthest from the amino group. On the other hand, the free amines aniline and 2-naphthylamine were oxidized to the *o*-hydroxy derivatives as well. 2-Naphthyl sulphamate appeared to behave in the same way as 2-acetamidonaphthalene in that hydroxylation occurred in the 6-position.

The metabolites identified from naphthalene were 1-naphthol and 1:2-dihydronaphthalene-1:2-diol, but 2-naphthol was not seen, and phenol was the only product identified from benzene.

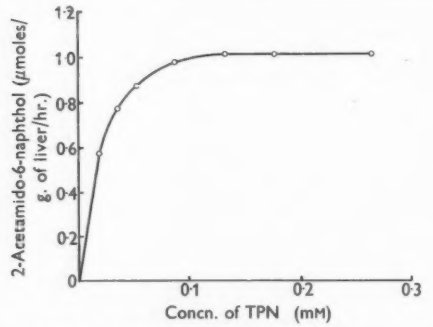


Fig. 2. Effect of TPN^+ concentration on the rate of hydroxylation of 2-acetamidonaphthalene. Reaction mixtures (3 ml.) contained 1 ml. of microsome preparation, 3 mM-G 6-P, 35 mM-nicotinamide, 2 mM-2-acetamidonaphthalene and 0·2 M-pyrophosphate buffer, pH 7·2, and were incubated for 30 min. at 38°.

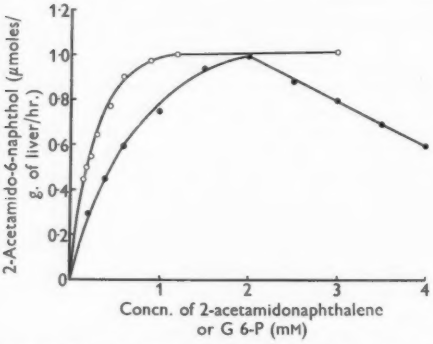


Fig. 3. Effect of G 6-P and substrate concentration on the rate of hydroxylation of 2-acetamidonaphthalene. Reaction mixture (3 ml.) contained 1 ml. of microsome preparation, 0·2 mM-TPN, 35 mM-nicotinamide and 0·2 M-pyrophosphate buffer, pH 7·2, and was incubated for 30 min. at 38°. ○, Hydroxylation with variations in G 6-P concentrations in the presence of 2 mM-2-acetamidonaphthalene; ●, hydroxylation with variations in 2-acetamidonaphthalene concentration in the presence of 3 mM-G 6-P.

DISCUSSION

Since Mueller & Miller (1948) found that a rat-liver homogenate required DPN and an oxidizable substrate for the hydroxylation of 4-dimethylaminoazobenzene in the 4′-position, the enzymic hydroxylation of several compounds has been reported, all the reactions requiring a reduced phosphopyridine nucleotide and oxygen.

The enzyme system responsible for the conversion of 2-acetamidonaphthalene into 2-acetamido-6-naphthol is present in the microsomes of rat liver and requires TPNH and oxygen. Apart from the buffer system employed the conditions are similar to those described by Mitoma *et al.* (1956) for the hydroxylation of acetanilide by rabbit-liver microsomes. Although these workers reported a maximum activity at pH 8·2 for the hydroxylation of acetanilide, whereas the pH optimum for 2-acetamidonaphthalene is 7·2, it would seem that the same enzyme system is involved, and qualitative experiments with some of the substrates used by Mitoma *et al.* (1956) such as aniline, acetanilide and naphthalene yielded essentially the same metabolites.

The mechanism of the hydroxylation reaction is still not understood, since it is difficult to explain the requirement for TPNH in an oxidative reaction. As aromatic compounds can be hydroxylated non-enzymically in the presence of H_2O_2 and Fe^{2+} ions (Loebl, Stein & Weiss, 1949) the possibility that H_2O_2 was involved has been investigated. Thus Mitoma (1956) found that H_2O_2-generating systems such as D-amino acid oxidase, uricase or xanthine oxidase and their respective substrates could not replace the DPN^+ or either of the liver fractions needed for the hydroxylation of phenylalanine; similarly the TPNH required for the hydroxylation of acetanilide could not be replaced by H_2O_2 generated by glucose oxidase or D-amino acid oxidase (Mitoma *et al.* 1956). Furthermore, neither of these reactions was inhibited by the addition of catalase, indicating that H_2O_2 was not involved. With $^{18}O_2$ and $H_2{}^{18}O$ it has been demonstrated that molecular oxygen, but not water, is utilized by the

Table 2. *Compounds hydroxylated by the rat-liver microsome preparation in the presence of TPN, nicotinamide and G 6-P*

Colours were developed by spraying paper chromatograms with the reagents described in Methods unless stated otherwise. Substrates were added in ethylene glycol monomethyl ether (0·1 ml.) to give the final concentration stated, but some of the less soluble compounds were present partly in suspension.

Substrate	Hydroxylated metabolite	Method of identification	R_F	Colour
Aniline (20 mM)	*o*-Aminophenol	Hexylresorcinol	0·82	Mauve
		2:6-Dichloroquinonechloroimide	—	Grey–green
	p-Aminophenol	Hexylresorcinol	0·71	Red
		1-Naphthol	—	Violet
Acetanilide (2 mM)	*p*-Acetamidophenol	1-Naphthol after hydrolysis of reaction mixture	—	Violet
		Indophenol test on hydrolysed reaction mixture	—	Blue
2-Naphthylamine (2 mM)	2-Amino-6-naphthol	Hexylresorcinol	0·85	Red
		Diazotized sulphanilic acid	—	Mauve
		NNCD reagent (acid)	—	Mauve
		NNCD reagent (alkaline)	—	Blue
	2-Amino-1-naphthol (identified by formation of 2-amino-1-naphthyl sulphuric acid)	Hexylresorcinol	0·56	Mauve
		p-Dimethylaminobenzaldehyde	—	Yellow
2-Acetamidonaphthalene (2mM)	2-Acetamido-6-naphthol	Diazotized sulphanilic acid	0·89	Red
		2:6-Dichloroquinonechloroimide	—	Blue
2-Naphthylsulphamate (20 mM)	6-Hydroxy-2-naphthyl sulphamate	Hexylresorcinol after hydrolysis	0·21	Red
		p-Dimethylaminobenzaldehyde after hydrolysis	—	Orange
		1-Naphthol after hydrolysis	—	Puce
2-Aminofluorene (2mM)	2-Amino-7-hydroxyfluorene	*p*-Dimethylaminobenzaldehyde	0·76	Orange
		Nitrite test on reaction mixture	—	Yellow
2-Acetamidofluorene (2 mM)	2-Amino-7-hydroxyfluorene	*p*-Dimethylaminobenzaldehyde	0·76	Orange
		Nitrite test on reaction mixture	—	Yellow
	2-Acetamido-7-hydroxyfluorene	Diazotized *p*-nitroaniline	0·79	Blue
		Nitrite test on hydrolysed reaction mixture	—	Yellow
Benzene (20 mM)	Phenol	2:6-Dichloroquinonechloroimide test on the reaction mixture	—	Blue
		NNCD reagent test on the reaction mixture	—	Red
Naphthalene (20 mM)	1-Naphthol	Diazotized sulphanilic acid	0·97	Red
		Diazotized *p*-nitroaniline	—	Blue
		2:6-Dichloroquinonechloroimide	—	Blue
	1:2-Dihydronaphthalene-1:2-diol	Diazotized sulphanilic acid after hydrolysis	0·86	Red
		Diazotized *p*-nitroaniline after hydrolysis	—	Blue
		2:6-Dichloroquinonechloroimide after hydrolysis	—	Blue

adrenal gland in the 11-β-hydroxylation of steroids (Hayano, Lindberg, Dorfman, Hancock & Doering, 1955). Further evidence for the utilization of molecular oxygen was provided by the finding that the oxygenation of the hydroxyl group which is introduced into salicylate by the model hydroxylase ferrous ion, ethylene diaminetetra-acetate, oxygen and ascorbate (Brodie, Axelrod, Shore & Udenfriend, 1954) arose from molecular oxygen (Mason & Onoprienko, 1956), and Mason, Fowlks & Paterson (1955) have shown that molecular oxygen contributes directly in phenolase-catalysed hydroxylations of some aromatic compounds. It has in fact been suggested (Velick, 1956) that phenolase may provide a model for the TPNH and oxygen-requiring systems, since if molecular oxygen and oxidizable metallo-enzymes were involved the TPNH would serve to regenerate the reduced and active enzyme. Both the hydroxylation of phenylalanine and acetanilide are inhibited by the ferrous ion-complexing agent, $\alpha\alpha'$-dipyridyl (Mitoma, 1956; Mitoma *et al.* 1956), whereas the 11β-hydroxylation of 11-deoxycorticosterone is strongly inhibited by diethyl dithiocarbamate (Sweat *et al.* 1956), suggesting that the enzymic activity in this case is associated with a copper complex.

Although naphthalene is converted into both 1-naphthol and 1:2-dihydronaphthalene-1:2-diol by this system, it seems likely that two different enzyme systems having the same location and requiring the same coenzyme are responsible for these two different types of reactions. 1:2-Dihydronaphthalene1:2-diol is readily dehydrated to 1-naphthol by mineral acid, but when it is incubated with the enzyme system no 1-naphthol can be detected, so that 1:2-dihydronaphthalene-1:2-diol is unlikely to be an intermediate between naphthalene and 1-naphthol. A further study of these two reactions may throw some light on the hydroxylation mechanism.

SUMMARY

1. The enzyme system responsible for the conversion of 2-acetamidonaphthalene into 2-acetamido-6-naphthol is associated with the microsomes of rat liver and requires reduced triphosphopyridine nucleotide (TPNH) and oxygen.

2. The TPNH can be replaced by triphosphopyridine nucleotide, glucose 6-phosphate (G 6-P) and G 6-P dehydrogenase, and conditions affecting the rate of hydroxylation of 2-acetamidonaphthalene by this sytem have been studied.

3. The system is not specific for 2-acetamidonaphthalene but will hydroxylate other aromatic amines, *N*-substituted amines and also benzene and naphthalene.

This work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research, Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

Bernstein, S. & McGilvery, R. W. (1952). *J. biol. Chem.* **198**, 195.

Booth, J., Boyland, E. & Manson, D. (1955). *Biochem. J.* **60**, 62.

Boyland, E. & Manson, D. (1955). *Biochem. J.* **60**, ii.

Boyland, E., Manson, D. & Orr, S. F. D. (1957). *Biochem. J.* **65**, 417.

Brodie, B. B. & Axelrod, J. (1948). *J. Pharmacol.* **94**, 22.

Brodie, B. B., Axelrod, J. Cooper, J. R., Gaudette, L., La Du, B. N., Mitoma, C. & Udenfriend, S. (1955). *Science*, **121**, 603.

Brodie, B. B., Axelrod, J., Shore, P. A. & Udenfriend, S. (1954). *J. biol. Chem.* **208**, 741.

Damron, C. M. & Dyer, H. M. (1953). *J. nat. Cancer Inst.* **14**, 279.

de Castro, F. T., Price, J. M. & Brown, R. R. (1956). *J. Amer. chem. Soc.* **78**, 2904.

Dobriner, K., Hofmann, K. & Rhoads, C. P. (1941). *Science*, **93**, 600.

Fincham, J. R. S. (1954). *J. gen. Microbiol.* **11**, 236.

Grant, J. K. & Brownie, A. C. (1955). *Biochim. biophys. Acta*, **18**, 433.

Hayano, M. & Dorfman, R. I. (1954). *J. biol. Chem.* **211**, 227.

Hayano, M., Lindberg, M. C., Dorfman, R. I., Hancock, J. E. H. & Doering, W. von E. (1955). *Arch. Biochem. Biophys.* **59**, 529.

Kensler, C. J., Miller, A. & Hyatt, M. (1956). *Proc. Amer. Ass. Cancer Res.* **2**, 124.

Loebl, H., Stein, G. & Weiss, J. (1949). *J. chem. Soc.* p. 2074.

Manson, L. A. & Young, L. (1950). *Biochem. J.* **47**, 170.

Mason, H. S., Fowlks, W. L. & Paterson, E. J. (1955). *J. Amer. chem. Soc.* **77**, 2914.

Mason, H. S. & Onoprienko, I. (1956). *Fed. Proc.* **15**, 310.

Mitoma, C. (1956). *Arch. Biochem. Biophys.* **60**, 476.

Mitoma, C., Posner, H. S., Reitz, H. C. & Udenfriend, S. (1956). *Arch. Biochem. Biophys.* **61**, 431.

Mitoma, C. & Udenfriend, S. (1955). *J. Pharmacol.* **113**, 40.

Mueller, G. C. & Miller, J. C. (1948). *J. biol. Chem.* **176**, 535.

Peters, J. H. & Gutman, H. R. (1956). *Arch. Biochem. Biophys.* **62**, 234.

Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 4951.

Ryan, K. J. & Engel, L. L. (1956). *J. Amer. chem. Soc.* **78**, 2654.

Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.

Sweat, M. L., Aldrich, R. A., de Bruin, C. H., Fowlks, W. L., Heiselt, L. R. & Mason, H. S. (1956). *Fed. Proc.* **15**, 367.

Udenfriend, S. & Cooper, J. R. (1952). *J. biol. Chem.* **194**, 503.

Velick, S. F. (1956). *Annu. Rev. Biochem.* **25**, 277.

Wiley, F. H. (1938). *J. biol. Chem.* **124**, 627.

The Study of Steady-State Concentrations of Internal Solutes of Mitochondria by Rapid Centrifugal Transfer to a Fixation Medium

By W. C. WERKHEISER* and W. BARTLEY
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

(*Received* 29 *October* 1956)

Reliable measurements of the distribution of solutes between mitochondria and their surrounding medium depends ideally on the rapid isolation of mitochondria from their medium. Moreover, it is essential that the solute composition of mitochondria should not change during this process. A method is described in this paper which deals with this problem. The mitochondria are centrifuged through a layer of silicone fluid immiscible with water, of a density intermediate between that of the particles and that of the medium. A high-density aqueous 'fixative' beneath the silicone can be used to stabilize labile compounds. Particles centrifuged in this manner carry with them an adherent film of their incubation medium. The amount of this adherent fluid can be measured by the inclusion of a radioactive high molecular (20 000–50 000) polysaccharide, such as inulin or polyglucose.

This paper describes the elaboration of this technique and its application to the study of the solutes of rat-liver mitochondria.

MATERIALS AND METHODS

Special chemicals

Silicone Fluid 702 [Edwards and Co., (London) Ltd.], sp.gr. 1·085, and Silicone Fluid M.S. 200/10 cs. (Hopkins and Williams Ltd.), sp.gr. 0·94, were mixed in suitable proportions to yield fluids of the required density. Silicone Fluid M.S. 1107 was prepared as a 2% (v/v) solution in CCl_4. Glassware to be rendered water-repellent was dipped in this solution, drained and heated at 110° for 2 hr.

Sodium adenosine triphosphate (ATP) was a commercial product (Zellstoff-fabrik Waldhof, Pharmazeutische Abteilung, Wiesbaden).

[^{14}C]Carboxypolyglucose (later referred to as 'labelled polyglucose') and [^{14}C]carboxyinulin were prepared by the following procedure based on the general method described by Isbell (1953). To a mixture of 1 m-mole of polymeric sugar and 1 m-mole of $NaHCO_3$ was added 1 m-mole of $K^{14}CN$ and 1 m-mole of KOH in a volume of 5 ml. or more if necessary. The mixture was held at room temperature until the reaction was complete. With polyglucose the time required was about 24 hr. whereas with inulin 3 days were necessary. The solution was heated in a water bath for 4–6 hr. at 80°, water being replaced as it was lost, and then dialysed overnight against water. In the preparation of polyglucose, 0·056 m-mole of each reagent, and in that of inulin, 0·014 m-mole, were used. No carrier KCN was used. (Polyglucose was presented by Dr P. T. Mora, National Cancer Institute, National Institutes of Health, Bethesda, U.S.A.) All other chemicals were analytical grade when available.

Homogenization media

Sucrose (0·25 M). In early studies sucrose was dissolved in the laboratory distilled water, chilled, and used directly. The pH of this solution was variable, but usually acid (pH 5–6) because of dissolved CO_2. The percentage dry wt. (see below) of mitochondria prepared in this acid sucrose was usually between 21 and 27 and they appeared visibly swollen when viewed in the phase-contrast microscope. Removal of most of the CO_2 from the water was accomplished by boiling for 10 min. in an open beaker, by prolonged evacuation on a water pump or by bubbling CO_2-free air through it for 2–3 hr. Sucrose was then dissolved in this water, the pH adjusted to 7·8 with aminotrishydroxymethylmethane (0·2 M) and the solution stored at 2° for not longer than 48 hr. The percentage dry wt. of mitochondria prepared in this medium was 31–34 and these mitochondria appeared quite dark and unswollen.

Modified sucrose solutions. Solutions of sucrose (0·25 M)–disodium ethylenediaminetetra-acetate (EDTA) (0·001 M), sucrose (0·25 M)–polyvinylpyrrolidone (PVP) (7·3%, w/v) and sucrose (0·25 M)–dextran (6%, w/v) were prepared in CO_2-free water and adjusted to pH 7·8 in a similar manner. PVP (L. Light and Co., Ltd.) was used as obtained or was dialysed exhaustively against distilled water. Dextran (gifts from Glaxo Laboratories Ltd., and Benger's Laboratories Ltd.) was used as received or after exhaustive dialysis against distilled water.

Mitochondrial fractionation

Sucrose mitochondria. These were prepared by the method of Schneider (1948) with minor modifications as suggested by the work of Appelmans, Wattiaux & Duve (1955). The complete fractionation procedure was performed at 0–2°. Chilled rat liver was homogenized in a stainless-steel Potter–Elvejhem homogenizer in 4 vol. of cold 0·25 M-sucrose solution and centrifuged for 10 min. at 2000 rev./min. (600 *g*) in rotor 822 of the refrigerated International Centrifuge (PR 2). The residue was resuspended in 1·5 vol. of sucrose and centrifuged for 10 min. at 1500 rev./min. (340 *g*). The combined supernatants were then centrifuged

* Present address: Roswell Park Memorial Institute, Buffalo, N.Y., U.S.A.

at full speed (4200 rev./min., 2700 *g*) for 20 min. The mitochondrial residue thus obtained was washed twice by resuspension in 1·5 vol. of sucrose and centrifuging for 10 min. in the 4-place high-speed head of the centrifuge at 2400 rev./min. (8500 *g*). All pinkish, freely flowing residue (fluffy layer) was discarded along with the supernatant in each of these decantations. It is worth emphasizing at this point that a voluminous fluffy layer appears only on washing the 'mitochondrial' sediment with pure sucrose solution. The sedimentation properties of both the true mitochondria and the fluffy layer are so modified in this medium that higher centrifugal forces are required to obtain a well-packed sediment. The presence of substances such as EDTA, PVP and dextran as well as 'supernatant' (see below) in the washing fluid greatly reduces the amount of fluffy layer and preserves the original sedimentation properties.

The final mitochondrial residue was resuspended in a small volume of sucrose and centrifuged at the full speed of the centrifuge (4200 rev./min. 26000 *g*) for 5 min. The supernatant was decanted, the tube well drained and the firmly packed residue was then resuspended in the desired incubation medium. The yield in a typical experiment was 4·4 g. wet wt. (1·5 g. dry wt.) of mitochondria/100 g. of liver.

Sucrose–EDTA mitochondria. The sucrose–EDTA medium was used in this preparation and the fractionation procedure outlined above was followed with one exception. The mitochondrial washings were performed in the large rotor 822 at full speed as in the first sedimentation of the mitochondria. As noted above, it was observed that in the presence of EDTA the usual fluffy layer did not form to any appreciable extent during washing, and lower centrifugal forces were needed to sediment the particles. During the washing procedure two distinct layers were observed in the sediment. The upper, somewhat pinkish layer was rather gelatinous and on gentle swirling could be poured off as an intact mass, leaving the buff-coloured mitochondrial layer behind.

Sucrose–PVP mitochondria and sucrose–dextran mitochondria. The preparation was as for sucrose–EDTA mitochondria.

'*Sucrose-supernatant mitochondria*'. The supernatant from the first mitochondrial centrifuging of a sucrose homogenate was further centrifuged for 1 hr. at 105000 *g* (30000 rev./min. in rotor 40) in the Spinco preparative ultracentrifuge, to remove remaining particles. The supernatant was stored in the deep freeze. 'Sucrose-supernatant mitochondria' were obtained by preparing sucrose mitochondria through the stage of the first sedimentation of the mitochondria. Washing of the mitochondria was then accomplished by the method for sucrose–EDTA mitochondria with 'supernatant' used as the washing medium.

Incubation conditions

The final mitochondrial pellet was resuspended in the chosen suspension medium at 0°. Labelled polyglucose [0·1 vol. of 9% (w/v) solution] was added plus any other desired components. The suspension was allowed to stand at 0° for at least 5 min. before the mitochondria were separated from their suspending medium by the centrifuging procedure.

Analytical methods

Chemical methods. Sodium and potassium were measured with a Beckman Flame Photometer, Model 9200. All phosphate determinations were made by the method of Berenblum & Chain (1938) as modified by Bartley (1953). Wet-ashing was performed by the method of Hanes & Isherwood (1949) with the addition of a drop of 5% (w/v) ammonium molybdate to facilitate the combustion. Sucrose was determined initially by the method of Bacon & Bell (1948), but later the method of Kulka (1956) was used, since it gave the more consistent results claimed by the author.

Chloride was determined by the method of Sanderson (1952). The Ag electrode was cleaned before use by brief immersion in 6N-HNO_3. With this cleaned electrode 0·1N-HNO_3 was used as the titration medium since the meter response was more rapid with this medium than with acetic acid.

Radioactivity determinations

Planchets for measurement of radioactivity were prepared on lens-tissue circles (16 mm.) centred on circular cover slips (19 mm.). Solutions containing trichloroacetic acid (TCA) were pipetted (0·05 ml.) on to planchets previously wetted with 0·01–0·02 ml. of a 10% (w/v) solution of mannitol in water. Solutions containing $HClO_4$ were pipetted (0·05 ml.) on to planchets which already contained 0·15 ml. of 0·4M-NaOH containing bromocresol purple. The liquid was stirred with a gentle stream of air until the colour of the indicator was uniformly purple. All planchets were dried at 60° in a small oven placed in a fume cupboard and then counted under a mica end-window counting tube. A minimum of 2500 counts were accumulated, which represents a standard error of less than 2%.

EXPERIMENTAL AND RESULTS

Whittam & Davies (1954) observed that the turnover of cellular sodium and potassium cannot be described by one rate constant. This observation suggests that more than one diffusion barrier exists in tissues and it has been thought that the mitochondrial membrane may represent such a second barrier. Bartley & Davies (1954), MacFarlane & Spencer (1953) and Stanbury & Mudge (1953) have in fact demonstrated concentration gradients between mitochondria and medium for several ions, dependent in at least one case (Bartley & Davies, 1954) on metabolism for their maintenance. In this work mitochondria had been separated for analysis by centrifuging, decantation and addition of acid to the pellet. It was recognized by Bartley & Davies (1954) that changes in the mitochondrial content of these labile compounds might occur in the time between sedimentation and addition of acid, but no way of avoiding this difficulty was then available. This problem does not occur in experiments with tissue slices, since the slices may be easily lifted out of their medium with forceps, blotted, and transferred to acid in a matter of seconds. The minimum time for the isolation of mitochondria by centrifuging, on the other hand, is a matter of minutes. This interval is indeed significant, for preliminary experiments showed that 19% of the endogenous organic phosphate of a mitochondrial suspension

Table 1. *Comparison of extraction of phosphate from mitochondria by various fluids*

To 1 ml. of the extracting fluid at 0° was added 1 ml. of a mitochondrial suspension in 0·25 M-sucrose containing 0·04 M-NaCl and 0·04 M-KCl. The extracting fluids contained the components listed in columns 1 and 2. The concentrations were such that the mixture with the mitochondria was of density 1·06 and contained either 0·5 M-$HClO_4$ or 10% (w/v) TCA. The phosphate is given as a percentage of that extracted by 10% (w/v) TCA alone.

Fixing acid	Density adjuster	Orthophosphate (%)	Organic phosphate (%)
TCA	Ammonium trichloroacetate	116	107
TCA	Urea	108, 101	105, 98·5
TCA	Formalin	94·5	98·8
$HClO_4$	Ammonium perchlorate	104	114
$HClO_4$	Urea	101	107
$HClO_4$	Formalin	96·5	97·7

sedimented at room temperature was hydrolysed in this brief time (about 5 min.). We have therefore developed a procedure for essentially simultaneous separation and 'fixation' by centrifuging mitochondria through a layer of silicone fluid immiscible with water into an aqueous acid 'fixative'. This acid must be of such a strength and nature that enzymic activities are very rapidly stopped, all unprecipitated solutes are extracted and the labile components of the extract are stabilized. For convenience we propose to call this process 'fixation' and the extracting fluid a 'fixative'. By this method the breakdown of organic phosphate before fixation was eliminated.

Treatment of centrifuge tubes. When two aqueous layers in a centrifuge tube are separated by a silicone layer, there is a tendency for the aqueous layers to become continuous down the walls of the tube, owing to the preferential wetting of the glass by the water. This was prevented by treatment of the tubes with M.S. 1107 silicone preparation, as described under Materials and Methods. The tubes then became water-repellent and complete isolation of the upper and lower water layers was achieved.

Choice of silicone density. A density differential of 0·015 between the mitochondrial suspending fluid and the silicone fluid was maintained. In addition, the overall density of the mitochondrial suspension was kept at least 0·007 below that of the silicone. Such a suspension contains not more than 25 mg. of dry mitochondria/ml.

Choice of fixative. The fixative must of course be of a density greater than the silicone above it. To achieve this density with conventional fixatives requires a concentration much greater than normally used [e.g. 20–30% (w/v) TCA or 2 M-$HClO_4$]. Two difficulties arise with the use of these reagents. TCA is extremely soluble in silicone fluids as well as water. In some manner not fully understood this results in a transfer of appreciable quantities of TCA and extracted mitochondrial contents to the fluid above the silicone upon centrifuging. Perchloric acid (2 M), although totally insoluble in silicone fluid, extracts more organic phosphate (about 16%) from the mitochondria than the generally accepted extracting fluids [e.g. 10% (w/v) TCA] and is thus also unsatisfactory. Search was therefore made for substances which would increase the density of the conventional extracting fluids [10% (w/v) TCA or 0·5 M-$HClO_4$]. Such materials should not increase the extraction of organic phosphates or be soluble in silicone, and ideally should not interfere with the subsequent analytical procedures. Table 1 summarizes the tests on various extraction fluids compared with 10% (w/v) TCA. On the basis of these results the ammonium salt mixtures were rejected.

The formalin–$HClO_4$ mixture (density 1·10) was used in the subsequent studies. The presence of so reactive a substance as formalin in the fixative is an obvious disadvantage as it may interfere with many analytical procedures (e.g. for sucrose or keto acids). Search for an inert substitute for formalin is proceeding.

Centrifuging procedure and recovery of mitochondrial phosphate in the extracting fluid. For separation of the mitochondria from their supernatant medium, the 6-place high-speed head of the centrifuge was used. The tubes were thick-walled (2 mm.) Pyrex glass of capacity 5·5 ml. A volume (1 ml.) of the selected fixative (when used) was pipetted into the bottom of the centrifuge tube, care being taken not to leave adherent drops on the wall. Silicone (1·5 ml., or 2·5 ml. when the fixative was omitted) of the appropriate density was layered over the fixative by means of a pipette whose tip was bent at a right angle. Finally 2 ml. of the mitochondrial suspension at 0° was added in a similar manner.

In preliminary experiments the control handle of the centrifuge was turned five divisions every 6 sec., reaching maximum in 1 min. Under these conditions (Table 2, line 1) a variable amount of the mitochondrial phosphate was found in the supernatant. Table 2 shows the effect of variation in the centrifuging procedure. Procedure 5 with the formalin–$HClO_4$ mixture (sp.gr. 1·10) was the most satisfactory in that the amount of mitochondrial

Table 2. *Influence of centrifuging procedure on the contamination of the supernatant fluid by mitochondrial phosphate*

The centrifuge tubes contained 2 ml. of mitochondrial suspension at 0° in 0·25 M-sucrose containing 0·04 M-KCl and 0·04 M-NaCl layered over 2·5 ml. of silicone or over 1·5 ml. of silicone beneath which was 1 ml. of fixative [containing either 0·5 M-$HClO_4$ or 10% (w/v) TCA]. The density of the fixative was adjusted to the indicated values with TCA, urea or formalin. The centrifuge was switched off after 2·5 min. running time. After centrifuging the supernatant was carefully stirred and a sample withdrawn for analysis of its phosphate content. Each number is the excess (μmoles) found in the tube with fixative over that found in the tube with silicone alone.

Nature of acid fixative	TCA					$HClO_4$				
Density adjuster	TCA	Urea		Formalin		Urea	Formalin			
Sp.gr.	1·15	1·08	1·10	1·06	1·13	1·06	1·06	1·08	1·10	1·11
Centrifuging procedure										
1. Handle turned 5 div. every 6 sec.	1·46	—	—	0·97	—	0·55	0·27	—	—	—
2. Handle turned 5 div. every 12 sec.	—	0·24	0·33	0·81	—	0·52	0·39	0·23	0·13	—
3. Handle turned in such a manner that the rate of increase of angular velocity was constant, reaching the maximum in 2 min.	—	0·32	0·46	—	—	—	—	0·06	0·09	—
4. Handle turned in such a manner that the rate of increase of centrifugal force was constant, reaching the maximum in 2 min.	—	0·33	0·49	0·41	0·19	—	—	0·16	0·11, 0·08	0·33
5. Handle turned to maximum as rapidly as possible	—	—	—	—	0·09	—	—	—	0·07, 0·02	0·20

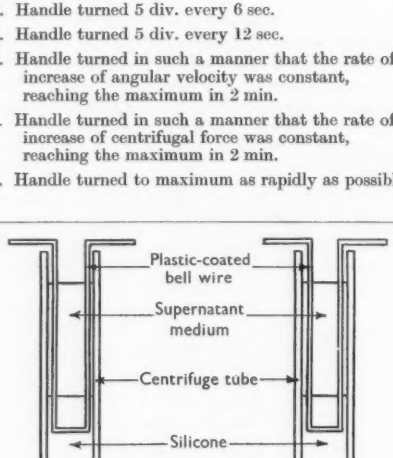


Fig. 1. Centrifuge tubes containing mitochondria sedimented through silicone fluid.

phosphate contaminating the supernatant in this case was less than 3% of the total acid-extractable phosphate. Table 2 also shows the very large amount of mitochondrial phosphate (approx. 50%) which can be transferred to the supernatant when concentrated TCA solutions are used as fixatives.

Although nearly all the mitochondrial phosphate was recovered in the formalin–$HClO_4$ extract under these conditions, varying amounts of the mitochondrial sodium, potassium and chloride were still transferred to the supernatant medium during centrifuging. Estimation of these latter stable ions was therefore made on pellets sedimented through silicone alone.

The solubility of TCA in silicone fluids suggested that this and other substances, dissolved in silicone, might serve as fixatives without any need for an aqueous layer. TCA, I_2 and SO_2 solutions in silicone were tested. All fixed the mitochondria, but at a rate too slow to be useful in the study of labile phosphates.

Final experimental technique. A pair of centrifuge tubes were required for a complete analysis of a mitochondrial suspension. In most cases duplicate pairs were actually prepared. In one tube of each pair was placed 2·5 ml. of silicone fluid of the appropriate density (1·05 when 0·25 M-sucrose was used as a suspension medium). In the other tube of each pair was placed 1 ml. of formalin–$HClO_4$ fixative (sp.gr. 1·10), the tube weighed and 1·5 ml. of silicone fluid added. All tubes were then placed in an ice bath and 2 ml. of mitochondrial suspension at 0° (containing 30–50 mg. of dry mitochondria) was layered over the silicone in each tube. The tubes were then placed in the chilled high-speed head and the refrigerated centrifuge was accelerated by turning the handle to maximum as rapidly as possible. After 2·5 min. the centrifuge was switched off and allowed to stop. The tubes were removed one at a time from the centrifuge head and into each was placed a U-shaped piece of plastic-coated bell-wire (Fig. 1). The ends of the wire were bent at right angles at such a level that when inserted in the tube the ends rested on the lip of the tube and the bottom of the U was 3–5 mm. within the silicone layer. The tubes were placed in a mixture of ethanol and solid CO_2 and left until it was convenient to continue the manipulations.

One of the tubes was removed from the bath and allowed to warm until the solid supernatant could be lifted from the tube by traction on the ends of the

wire. The surface of the solid supernatant was wiped free from the adherent silicone with filter paper. The supernatant was suspended by means of the wire on the rim of a weighed 15 ml. conical centrifuge tube containing 1 ml. of 30 % (w/v) TCA and, on thawing, it dripped into the TCA and was deproteinized. On reaching room temperature the tubes were weighed and the contents mixed. After centrifuging to remove precipitated protein the solutions were decanted and stored at 2°.

As soon as the supernatant had been removed from the tube the liquid silicone was poured off, leaving either the $HClO_4$ extract or mitochondrial pellet still frozen. The bottom of the tube was pushed through a hole in a rubber stopper into a container of solid CO_2 (Fig. 2) and the whole inverted and allowed to drain. After about 30 sec. the inside rim of the tube was wiped with filter paper, and the tube removed from the container, filled with hexane at room temperature and returned to the ethanol–CO_2 bath. When thoroughly chilled the hexane was decanted and the tube allowed to drain while in the solid-CO_2 container. Two further hexane washings were necessary to remove all but traces of silicone from the tube.

After the last hexane washing the tube containing the formalin–$HClO_4$ extract was stoppered and allowed to warm to room temperature. The mitochondrial pellet in the tube without fixative usually became loose after the first hexane washing and was allowed to drop on filter paper, where excess of hexane rapidly evaporated without melting of the pellet. At this point the pellet was picked up with clean forceps and returned to its centrifuge tube, which was filled with hexane. After the third hexane washing the dried frozen pellet was put into a similar weighed centrifuge tube containing an accurately weighed portion of 10 % (w/v) TCA (1 ml.), and the tube stoppered. On occasions when the total dry wt. and the water content of the pellet were to be determined, the pellet was placed in a clean, weighed tube. When the tubes had attained room temperature the stoppers were removed and the remaining traces of hexane were allowed to evaporate. The tubes were then weighed after removal of moisture from the outside.

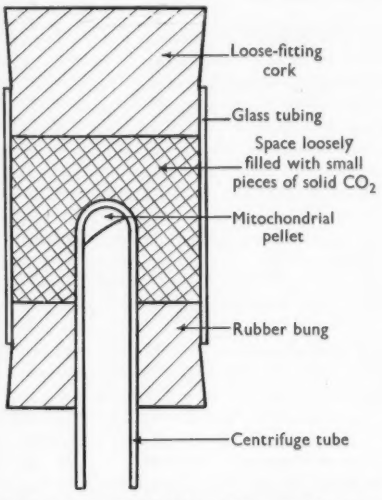


Fig. 2. Apparatus for draining silicone and hexane from frozen mitochondrial pellets.

The mitochondrial pellets in 10 % (w/v) TCA were broken into small fragments with a glass rod. The tubes containing TCA and formalin–$HClO_4$ were stored overnight at 2° and those containing the unextracted pellets were dried overnight in an oven at 105°.

The next morning the contents of the tubes containing the extracted pellets were stirred and the tubes centrifuged at 0° for 5 min. The extracts were decanted and stored at 2° to await analysis. The residues were reserved for determination of mitochondrial dry wt. as described in the next paragraph.

The tubes containing the dried pellets were cooled and weighed and the percentage dry wt. of the pellet was calculated.

Determination of 'acid-insoluble mitochondrial dry matter'. The TCA-extracted residues, after decantation of the extracting fluid, were washed once with 2 ml. of 5 % (w/v) TCA and once with 2 ml. of water. The residues were separated from the washing fluid each time by centrifuging for 5 min. at the full speed of the high-speed head of the centrifuge. Water (1 ml.) was added to the residue and the tubes were dried in an oven at 105° overnight. During this time remaining traces of TCA decomposed. The tubes containing the residues were then cooled and weighed. If to the weights of the acid-insoluble mitochondrial dry matter (subsequently referred to as M) so obtained was added the weight of sucrose and other solutes contained in the TCA extract, the sum agreed to within less than 1 % with that obtained by directly drying a similar mitochondrial pellet.

In some of the early experiments, the TCA washing of the residue was followed by two washings with 2 ml. of ethanol before drying in the oven. It was later discovered that these brief washings with ethanol removed about 10 % of the acid-insoluble solids. These results have therefore been corrected for this error. Since this correction is an approximation, the resulting values for M may have errors of as much as 5 %.

Dry weight of sedimented mitochondrial pellet. For purposes of comparison with mitochondria sedimented through silicone, a series of determinations were made on the dry wt. and water content of pellets centrifuged directly from mitochondrial suspensions at 0° without the use of silicone. The tubes were removed from the centrifuge and the supernatant was decanted. Then, with the tube maintained in an inverted position the internal walls of the tube were carefully dried with filter paper. Great care was taken to avoid touching the pellet. The tube containing the pellet was weighed, dried overnight at 105°, cooled and reweighed. These two weighings supplied the data for the calculation of the percentage dry wt. of the pellet.

Estimation of extra-particulate fluid. The accurate analysis of internal solute concentrations in mitochondria requires the estimation of the amount and solute content of the extra-particulate fluid of the pellet. The amount of this adherent medium may be determined from the distribution between the pellet and the medium of a high-molecular-weight substance, which neither penetrates the mitochondria nor is adsorbed on their surface. Polyglucose (Pacsu & Mora, 1950; Mora & Pacsu, 1955), of a molecular weight of 50000, is uncharged, spherical, very soluble in water, but only slightly hydrated. These properties suggest that it would be at least as suitable as inulin for the estimation of extra-particulate fluid.

Accordingly the distribution of polyglucose, inulin and sucrose in mitochondrial pellets sedimented from different media was investigated. For ease of analysis in the presence of sucrose the polyglucose and inulin were tagged with a terminal [^{14}C]carboxyl group. Sufficient labelled polyglucose was found to be present in the mitochondrial pellet sedimented from a suspension of mitochondria in sucrose to account for 19–22 % of the total water of the pellet (Table 3). Since it seemed unlikely that the extra-particulate space could be much smaller than this, and that the use of polyglucose could lead to an underestimation, we have assumed that the distribution of labelled polyglucose in fact measures the extra-particulate fluid of the pellet and have included it in the medium in all further experiments in which this fluid was to be measured. Inulin has been extensively used as a measure of extracellular space in whole animals and tissue slices. Since inulin is not easily estimated in the presence of

Table 3. *Distribution of carboxypolyglucose, carboxyinulin and sucrose in mitochondrial pellets*

Volumes (2 ml.) of the indicated mitochondrial suspensions at 0° were centrifuged through silicone, the pellets washed with hexane and extracted with 10 % (w/v) TCA. The supernatants and extracts were assayed for the three compounds.

Medium used for preparation and suspension of mitochondria	Expt. no.	Total water (%) 'penetrated' by			Internal water 'penetrated' by sucrose (%)
		Carboxy-polyglucose	Carboxy-inulin	Sucrose	
Sucrose	1	19·4	—	67	58
		20·1	—	66	57
		19·3	—	66	58
	2	22·2	—	68	58
		20·6	—	67	57
		20·5	—	69	59
Sucrose–EDTA	3	27·0	—	70	58
		26·5	—	69	57
	4	19·5	—	64	55
		18·1	—	63	55
Sucrose–EDTA + salt*	3	36·2	—	77	64
		33·2	—	76	64
		—	65	—	—
		—	68	—	—
Sucrose–EDTA + salt*	4	27·3	—	73	63
		25·3	—	70	60
Sucrose–PVP	5	44·7	—	77	56
		42·8	—	78	62
Sucrose–PVP + salt*	5	41·4	—	76	60
		40·4	—	77	63
Dialysed sucrose–PVP	6	30·8	—	82	74
		30·6	—	81	72
Dialysed sucrose–PVP + salt*	6	33·8	—	75	63
		31·4	—	76	64

* Salt indicates that the medium contained 0·04 molal KCl and 0·04 molal NaCl.

sucrose the distribution of [^{14}C]carboxyinulin was measured in a mitochondrial pellet sedimented from sucrose–EDTA solution. The results (Table 3) suggest that inulin had penetrated about two-thirds of the water of the pellet, although labelled polyglucose in a similar preparation had penetrated only one-third of this water. Thus the mitochondria appear to be appreciably permeable to carboxyinulin. Whether this is due to true penetration or merely to adsorption is not clear.

Permeability of mitochondria to sucrose. Although sucrose has been used by most workers in fractionation of cell particulates, its function remains obscure. It has generally been assumed that it does not penetrate the mitochondria, as measurements of the volume changes of mitochondria in sucrose solutions of varying strength indicate that they act as osmometers (for example, see Tedeschi & Harris, 1955). The data for sucrose in Table 3 indicate that this solute penetrates a large proportion of the intramitochondrial water. The amount of intramitochondrial sucrose is sufficient to justify the assumption that 57–59 % of the intramitochondrial water had the same sucrose concentration as the external medium. In other words, the 'sucrose space' within the mitochondria was about 60 % of the total water space.

Table 3 also shows that the internal sucrose space of sucrose–EDTA mitochondria was the same as that of sucrose mitochondria in spite of varied polyglucose spaces. The sucrose space of the mitochondria was appreciably increased by the addition of NaCl and KCl at final concentrations in the medium of 0·04 molal (Table 3, Expts. 3 and 4).

In Table 3 are also shown the polyglucose and sucrose 'spaces' of mitochondria isolated from PVP media. In Expt. 5 it was observed that the sucrose space, both in the presence and absence of KCl (0·04 molal) and NaCl (0·04 molal), was similar to that in sucrose–EDTA plus the same salt mixture. In Expt. 6, the use of dialysed PVP resulted in a greatly increased sucrose space in the salt-free medium. This space was reduced on the addition of NaCl and KCl to the medium, suggesting that the salt content of the undialysed PVP was responsible for the somewhat lower values in Expt. 5 before the medium had been supplemented with the salt mixture.

Calculation of internal solute concentration of mitochondria. The weight of the mitochondrial pellet may be considered to be composed of four separate weights: that of the extracted, washed and dried mitochondrial residue (M), that of its soluble internal solutes (S), that of its internal water (I) and that of the adherent supernatant fluid (E).

Calculation of the amounts of acid-soluble solutes in the mitochondrial pellet ($S+E$) requires the estimation of the volume of the acid extract of the pellet. This was taken to be the volume of the TCA (1 ml.) + the wt. of the mitochondrial pellet ($\sim$0·15 g.) $-M$ ($\sim$0·04 g.). No correction has been made for the density of the fluid content of the pellet, which must be similar to that of the suspending medium (1·035–1·050). The error due to this approximation is small ($\sim$0·5 %).

The volume of E was calculated from the measurements of radioactivity in the mitochondrial extract and the supernatant fluid. The measurement of the density of the medium allowed calculation of the weight of E.

The total content of the various solutes of the mitochondrial extract was calculated from the results of the chemical determinations. The concentrations of the same solutes in the supernatant fluid were also determined, and thus the contribution of adherent medium to the total solute content of the pellet was calculated from the known volume E. Subtraction of these amounts yielded the amounts of the internal mitochondrial solutes (S). (In this calculation the molecular weight of the organic phosphates was assumed to be approx. 300.) As not all soluble components of the mitochondria were measured, S must be low; however, by independent measurement it was found that this deficit amounted to no more than 0·3 mg./100 mg. wet wt. of mitochondria. This slight deficit was ignored. It should be noted that S is only about 3 % of the total weight of the mitochondrial pellet and largely comprises sucrose.

From the total weight of the mitochondrial pellet and the values of M, S and E, the weight (and volume) of the internal water (I) was calculated by difference.

The concentrations of the individual internal solutes making up S were obtained by dividing by I. These concentrations are thus expressed on a millimolal basis.

It should be noted that the mitochondria spun into the formalin–$HClO_4$ fixative were used only for the determination of labile compounds (phosphates), as the remainder of the solutes could only be reliably estimated on the TCA extracts.

The indirect calculation of I described above has been found to agree to within 1 % with that obtained by measuring the water content of $I+E$ (loss in weight on drying of the mitochondrial pellet) and deducting the contribution of E.

Water content of mitochondria. To our knowledge there are no published values for the water content of mitochondria prepared and maintained in 0·25 M-sucrose at 0°. Knowledge of this value would appear essential for evaluation of the degree of swelling occurring when these mitochondria are suspended in other media or at elevated temperatures.

Therefore measurements were made on mitochondria at 0° prepared in 0·25 M-sucrose and resuspended in 0·25 M-sucrose with or without the additions given in Table 4. The percentage dry weights of sedimented mitochondrial pellets were determined by drying at 105°. Table 4 shows that whether or not the mitochondria passed through silicone the percentage dry wt. of the pellet was the same. All the preparations studied yielded a pellet with a percentage dry wt. greater than 32. The only published values for mitochondrial dry wt. (MacFarlane & Spencer, 1953; Price & Davies, 1954; Fonnesu & Davies, 1955; Klemperer, 1955) are of the order of 20 %. The mitochondria studied by these workers were under their conditions already swollen to twice the water content that they presumably had when they were at 0° in sucrose alone.

The addition of 0·1 vol. of a 9 % (w/v) labelled polyglucose solution to a suspension of mitochondria in 0·25 M-sucrose, with or without the addition of NaCl and KCl to give final concentrations of about 0·04 molal, caused a slight extrusion of water from the particles (5–10 %). This is probably due to the colloid osmotic pressure exerted by the polyglucose and is additional evidence that this substance does not penetrate the mitochondria.

It is apparent that the dry wt. of the pellet as presented above will vary with the amount of solutes dissolved in the intramitochondrial and the extraparticulate fluids of the pellet. Thus changes in percentage dry wt. (as defined above) incident on changes in medium or conditions may give erroneous impressions of shrinking or swelling. The measurement of the interstitial fluid by the use of labelled polyglucose enables the internal mitochondrial water (I) and the non-extractable solids (M) to be calculated. The ratio of these two values, I/M, is a more sensitive and meaningful estimate of mitochondrial hydration permitting comparison of mitochondria in widely differing suspension media.

Solute content of mitochondria prepared in 0·25 M-sucrose at 0°

While a fairly consistent pattern for the content of some solutes of mitochondria at 0° was observed, others were subject to considerable variation. Table 5 gives the values observed in three representative experiments. The three most consistent of the components studied were potassium, orthophosphate and organic phosphate. Chloride was subject to great variation and may be present in the mitochondria only as a contaminant. Although the external concentrations of sodium and potassium were similar (about 0·7 millimolal), the particles retained about 10 times more potassium than sodium (50 millimolal K, 6 millimolal Na). A large and consistent proportion of internal phosphate

Table 4. *Percentage dry weight of centrifuged mitochondrial pellets*

Volumes (2 ml.) of mitochondrial suspensions at 0° in 0·25 M-sucrose containing the indicated additions were centrifuged for 5 min. at 25 000 *g*. Some of the suspensions were layered over 2·5 ml. of silicone. The concentration of labelled polyglucose was 0·9 %, of KCl 0·04 M, of NaCl 0·04 M. When silicone was present the tubes were washed with hexane by the procedure described in the text. When silicone was absent the supernatant was decanted and the tube wiped with filter paper. After the residues had been weighed they were dried overnight at 105° and reweighed. The values in the table are weight of dry residue × 100/wt. of wet residue.

Additions			No silicone			Silicone		
Labelled polyglucose	KCl	NaCl	% dry wt.	S.E.M.	No. of expts.	% dry wt.	S.E.M.	No. of expts.
−	−	−	33·9	0·3	(10)	.	.	.
+	−	−	35·3	0·3	(4)	.	.	.
−	+	−	32·5	0·2	(18)	32·7	0·3	(14)
−	+	+	33·0	0·4	(8)	32·3	0·7	(8)
+	+	+	.	.	.	34·5	0·2	(16)

Table 5. *Concentrations at 0° of internal solutes of mitochondria prepared in sucrose solutions*

Samples (2 ml.) of mitochondrial suspensions at 0° were sedimented through silicone as described in the text. All preparations contained labelled polyglucose. I, Weight of internal water; M, weight of dry matter in mitochondria.

	Millimolal						
Expt. no.	K	Na	Cl	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
1	47	5	32	6·8	36	84	—
2	48	4	17	8·2	44	84	1·91
3	59	9	1	7·5	39	84	1·63
External concn.	0·8	0·6	2·3	0·10	0·26	—	—

(84 %) was in the form of organic esters, roughly half of which were adenine nucleotides. The total concentration of the phosphate esters was somewhat less than that of the potassium.

Solute content of mitochondria prepared in other media at 0°

Sucrose–EDTA. EDTA, according to Beaufay, Hers, Berthet & Duve (1954) and Slater & Cleland (1953), appears to stabilize certain mitochondrial enzymes. It has been widely used and is generally assumed to have no undesirable effects. The solute composition of mitochondria prepared in and separated from 0·25 M-sucrose containing 0·001 M-EDTA at 0° is shown in Table 6. As in sucrose mitochondria, the chloride concentration showed considerable variation and in this case potassium, although lower, was also somewhat variable. The organic phosphate concentration was somewhat lower (31 millimolal) but the percentage organic phosphate was much higher (92 %). Thus the most striking difference from sucrose mitochondria was the low orthophosphate concentration. The water content (as indicated by the values of I/M) appeared somewhat higher.

Sucrose–PVP. Novikoff (1956) has described the use of PVP as an addition to sucrose in the preparation of mitochondria. He states that mitochondria prepared in this medium retain the thread-like shape characteristic of their intracellular condition. Electron-microscope studies have shown that the mitochondria in the sucrose–PVP medium retain the *cristae mitochondriales* intact. Dalton (1957) showed that in mitochondria prepared in 0·25 M-sucrose the *cristae* were frequently ruptured; as might be expected in view of this finding, mitochondria isolated from 0·25 M-sucrose are not rod-like but tend towards a spherical shape. In contrast, mitochondria from tissues homogenized in 0·25 M-sucrose containing 7·3 % of PVP appeared rod-like when observed with phase-contrast microscopy. The solute concentrations of mitochondria prepared in PVP media at 0° are given in Table 7. In the third experiment the PVP was exhaustively dialysed against distilled water before use. The retention of potassium was much less by the PVP mitochondria than by the sucrose mitochondria (average about 20 millimolal in PVP against about 50 millimolal in sucrose). The potassium retention was even less when the PVP had been dialysed. As was previously observed for the EDTA mitochondria the orthophosphate concentration was only about one-third of that found in the sucrose mitochondria. The ratio of organic phosphate to total phosphate did not show the same constancy exhibited by the sucrose mitochondria.

The water content as indicated by the values of $I:M$ give no support to the concept that the PVP mitochondria are less swollen than sucrose mitochondria. It would appear that rupture of the *cristae* involves a change in shape without a marked change in hydration.

Dextran mitochondria. The possibility that PVP exerted its action by virtue of its colloidal nature suggested that other similar substances might be used for keeping mitochondria in the rod-shaped state. Dextran is one such substance, and Hogeboom (1955) has stated that liver mitochondria

Table 6. *Concentrations at 0° of internal solutes of mitochondria prepared in sucrose–EDTA solutions*

Details are as in Table 5.

	Millimolal						
Expt. no.	K	Na	Cl	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
1	27	4	21	1·9	32	94·5	2·18
2	36	8	39	3·2	30	90	1·96
External concn.	0·8	0·7	0·3	0·10	0·29	—	—

Table 7. *Concentrations at 0° of internal solutes of mitochondria prepared in sucrose–PVP solutions*

Details are as in Table 5. In Expt. 3 the PVP was exhaustively dialysed before use.

	Millimolal						
Expt. no.	K	Na	Cl	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
1	15	8	39	1·5	45	97	1·81
2	25	8	34	0·9	50	98	1·75
3	7	6	27	2·2	28	93	1·82
External concn.	2·0	0·8	0·3	0·27	0·83	—	—

prepared in isotonic sucrose containing 6–12 % of dextran retain their normal rod-like shape (see also Birbeck & Reid, 1956). Mitochondria isolated from 0·25 M-sucrose containing 6 % of dextran at 0° were not rod-like under our conditions. From the analysis of the external medium it appears that the undialysed dextran contained an appreciable amount of NaCl. The potassium retention in dextran medium was very low (Table 8). Orthophosphate concentration was also lower than in sucrose mitochondria, but more striking was the low concentration of the organic phosphates (about 23 millimolal). It is uncertain whether the swollen state of the mitochondria in the second experiment (I/M 3·66) was related to the fact that the dextran was undialysed.

'*Supernatant*' *mitochondria.* The cell sap may contain cofactors which play a part in preserving the morphological and physiological integrity of the mitochondria in the intact cell. The loss of these substances on washing with sucrose solutions may be the cause of the appearance of the characteristic 'fluffy' layer and perhaps cause other changes in the mitochondria themselves. Table 9 contains data of the solute concentrations of mitochondria washed with and resuspended in 'sucrose supernatant' prepared as already described. The sodium, potassium and chloride values resemble those for PVP mitochondria much more than those for sucrose mitochondria, but the organic and orthophosphate values are similar to those for sucrose mitochondria. Although this preparation was grossly swollen, the value for organic phosphate was not reduced; thus the content of organic phosphate was 50 % greater than in any of the other preparations. Phosphate loss thus appears to be reduced in this medium.

Solute distribution in mitochondria at 0° *in the presence of* NaCl *and* KCl. In the previous section we have described the retention of simple ions by thoroughly washed mitochondria at 0°. These ions therefore are bound under this set of circumstances. On the addition of a mixture of NaCl and KCl, the concentrations of sodium, potassium and chloride are appreciably higher in the mitochondrial water than in the medium. The excess amount (m-moles/kg. of M) of such ions is roughly the same as was present in the mitochondria before the addition of the salt, indicating that these ions are still bound. Table 10 illustrates this finding with respect to potassium. Similar results were obtained for the distribution of added sodium and chloride. The addition usually caused a shrinkage (10–25 %) of the mitochondria. In three of the media shrinkage did not occur. In the dialysed PVP medium there was no change in hydration and with the dialysed dextran and 'supernatant' media swelling occurred on the addition of the salt mixture.

Effect on the internal phosphates of the addition of nucleotides at 0°. To suspensions of mitochondria at 0° to which salt (final molal concentration of NaCl and KCl was 0·04) had been added was subsequently added a solution of a commercial preparation of adenosine triphosphate (ATP) to raise the total external phosphate concentration to 0·01–0·015 molal. This nucleotide solution contained (on a phosphate basis) 44 % of ATP, 31 % of adenosine diphosphate, 7 % of adenosine monophosphate, 6 % of orthophosphate and 12 % of unidentified phosphates. As shown in Table 11, the addition of

Table 8. *Concentrations at* 0° *of internal solutes of mitochondria prepared in sucrose–dextran solutions*

Details are as in Table 5.

Expt. no.	Type of dextran used	Millimolal K	Na	Cl	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
1	Benger's (dialysed)	7	5	46	2·7	23	90	1·85
External concn.		1·0	0·6	0·8	0·2	0·6	—	—
2	Glaxo (undialysed)	9	14	31	4·7	20	82	3·66
External concn.		0·6	4·7	3·1	0·12	0·35	—	—

Table 9. *Concentrations at* 0° *of internal solutes of mitochondria prepared in* '*supernatant*'

Details are as in Table 5.

Expt. no.	Millimolal K	Na	Cl	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
1	21	9	35	6·3	48	88	2·45
External concn.	13	5	5	1·6	4	—	—

this nucleotide solution caused a large increase in orthophosphate concentration of sucrose mitochondria. Concomitantly the percentage of organic phosphate decreased, although there was no decrease in organic phosphate and the water content showed only slight changes. In contrast the addition of the nucleotide mixture to sucrose–PVP mitochondria in the salt-containing medium caused only a slight increase in intramitochondrial orthophosphate concentration, and thus had no effect on the proportion of organic phosphate, but caused the mitochondria to swell by about 20 %.

Effect of 2:4-dinitrophenol (DNP) on mitochondrial solutes at 0°. As DNP has been used in many investigations on tissue preparations it was of interest to investigate its effect on the internal solutes of mitochondria. Table 12 shows the effect of the addition of DNP (2×10^{-4} M) to a suspension of mitochondria at 0° in 0·25 M-sucrose and in 0·25 M-sucrose containing 0·04 molal KCl and 0·04 molal NaCl. In both cases this addition resulted in a marked loss of 'bound' potassium and a concomitant loss in phosphates. The phosphate loss from mitochondria isolated from the sucrose medium was equally contributed to by organic and orthophosphate. The phosphate loss from mitochondria isolated from the salt-containing medium was apparently confined to orthophosphate.

The effect of DNP on mitochondrial hydration appears to vary according to the experimental conditions (MacFarlane & Spencer, 1953; Price & Davies, 1954; Klemperer, 1955). Under our conditions, at 0°, DNP added to a mitochondrial suspension in 0·25 M-sucrose caused the same degree of shrinkage as that which occurred on the addition of the salt mixture to the same suspension (cf. line 1

Table 10. *Effect of salt on the binding of potassium by mitochondria at 0°*

Details are as in Table 5. Salt, when added, raised the external concentration to about 0·04 molal KCl and 0·04 molal NaCl. Excess concentrations represent the numerical difference in concentration between mitochondria and medium. M, mitochondrial dry wt.

	Excess concn. (millimolal)		Excess content (m-moles/kg. of M)		Relative water content (I/M)	
Mitochondrial suspension medium	Without salt	With salt	Without salt	With salt	Without salt	With salt
Sucrose	46	42	—	—	—	—
Sucrose	47	57	90	97	1·91	1·71
Sucrose	58	53	95	77	1·63	1·45
Sucrose	—	45	—	77	—	1·70
Sucrose–EDTA	26	29	57	46	2·18	1·60
Sucrose–EDTA	35	44	87	53	1·96	1·50
Sucrose–PVP	13	28	23	45	1·81	1·62
Sucrose–PVP	23	29	40	43	1·75	1·47
Sucrose–dialysed PVP	5	0	9	0	1·82	1·82
Sucrose–dextran (Benger's dialysed)	6	1	11	2	1·85	2·14
Sucrose–dextran (Glaxo undialysed)	8	8	29	24	3·66	2·96
'Supernatant'	8	10	20	28	2·45	2·79

Table 11. *Effect of 'ATP' on the internal phosphate of mitochondria at 0°*

Details are as in Table 5. All suspensions contained approx. 0·04 molal KCl and 0·04 molal NaCl. The 'ATP' preparation was that described in the text. M, mitochondrial dry wt.

			Millimolal		m-moles/kg. of M			
Suspension medium	Additions	Fluid analysed	Ortho-phosphate	Organic phosphate	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
Sucrose	None	Mitochondrial	6·1	41	10·2	68	87	1·67
		Medium	0·06	0·2	—	—	—	—
	'ATP'	Mitochondrial	17	42	28	69	72	1·65
		Medium	1·2	10·9	—	—	—	—
Sucrose	None	Mitochondrial	8·0	44	13·6	75	85	1·70
		Medium	0·05	0·2	—	—	—	—
	'ATP'	Mitochondrial	20	50	35	88	72	1·75
		Medium	1·5	11·7	—	—	—	—
Sucrose–PVP	None	Mitochondrial	3·0	41	4·9	67	93	1·62
		Medium	0·01	1·0	—	—	—	—
	'ATP'	Mitochondrial	3·9	44	7·5	85	92	1·93
		Medium	0·3	17·8	—	—	—	—

Table 12. *Effect of 2:4-dinitrophenol on the internal potassium and phosphates of mitochondria at 0°*

Details are as in Table 5. Mitochondria were prepared and suspended in 0·25 M-sucrose. M, mitochondrial dry wt.

Additions	Mitochondrial content (m-moles/kg. of M)			External concn. (millimolal)		Relative water content (I/M)
	Excess K*	Content of Ortho-phosphate	Content of Organic phosphate	Ortho-phosphate	Organic phosphate	
None	95	12·2	64	0·09	0·27	1·63
2×10^{-4} M-DNP	59	8·0	59	0·28	0·33	1·40
0·04 M-KCl + 0·04 M-NaCl	77	10·4	61	0·08	0·23	1·45
0·04 M-KCl + 0·04 M-NaCl + 2×10^{-4} M-DNP	56	3·2	61	0·32	0·25	1·69

* Calculation as in Table 10.

with lines 2 and 3, Table 12). The addition of DNP to a mitochondrial suspension containing the salt mixture, on the other hand, caused a swelling of the same order as the previous shrinkage.

DISCUSSION

Knowledge of the solute contents of mitochondria at 0° before possible degenerative changes is essential to an understanding of the effects of various experimental conditions at elevated temperatures. It is worth emphasizing that all the studies reported here were conducted at 0°.

Throughout this work the assumption has been made that the internal mitochondrial solution represents a single aqueous phase. Our calculations are thus based on the even distribution of the internal solutes within the internal water. The distribution of added potassium, sodium and chloride appears to be in agreement with this assumption, but the partition of sucrose suggests that the whole of the mitochondrial water may not be available to this substance. The fact that the internal sucrose concentration is considerably less than the external would, on the basis of a single aqueous phase, imply a mechanism for the partial exclusion of sucrose. However, an inert uncharged molecule such as sucrose would be expected to be passively distributed, and an alternative explanation for these results would be that there are two internal aqueous phases, the concentration of sucrose in the more accessible of these compartments being the same as that in the external medium. The apparent partial penetration of mitochondria by sucrose could also be explained on the basis of varying permeability of the mitochondrial population. Thus two sorts of mitochondria might be visualized, one completely permeable to sucrose and the other impermeable. It is impossible as yet to distinguish between the various possibilities. The observation that the sucrose space of mitochondria prepared in dialysed sucrose–PVP medium was reduced on the addition of a mixture of sodium, chloride and potassium chloride seems to favour the hypothesis that part of the mitochondrial water is impermeable to sucrose.

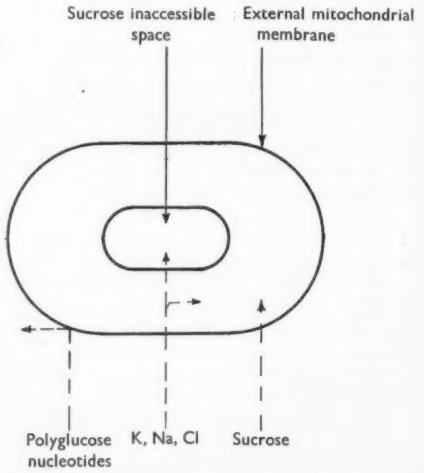


Fig. 3. Diagrammatic representation of the permeability of mitochondria to various solutes. The broken lines show areas of the mitochondria accessible to the solutes.

Our experiments show that the addition of a nucleotide mixture to a mitochondrial suspension produced no change in the internal organic phosphate concentration. This may be due to the impermeability of the mitochondrial membrane to these nucleotides. Fig. 3 summarizes these observations in diagrammatic fashion.

Our results indicate that quantitatively the chief intraparticulate ions retained in washed mitochondria are potassium and phosphates. Thus mitochondria appear to exhibit the same selectivity for potassium and phosphates as intact cells when incubated in a saline medium. However, the quantities retained by these washed mitochondria are small compared with the total intracellular

content, and it is therefore impossible at present to conclude that the mitochondria are responsible for the ionic selectivity of whole cells.

SUMMARY

1. Rapid isolation of rat-liver mitochondria from their suspending medium has been achieved by centrifuging them into a layer of silicone fluid. A high-density aqueous 'fixative' beneath the silicone stabilized labile phosphate esters when these were to be measured. In the absence of this fixative 19% of the mitochondrial acid-soluble organic phosphate was hydrolysed in 5 min. at room temperature.

2. In this procedure the centrifuge tubes were cooled in a solid carbon dioxide–ethanol bath, the frozen aqueous supernatant fluid was removed, the silicone poured off, and residual silicone removed from the lower layer by washing with hexane at low temperature.

3. The volume of extra-particulate fluid in the mitochondrial pellets as estimated from the distribution of radioactive polyglucose between pellet and medium represented 19–22% of the total water of mitochondrial pellets isolated from 0·25M-sucrose solutions at 0°.

4. Mitochondria were found to be permeable to sucrose. The internal concentration of sucrose in mitochondria isolated from 0·25M-sucrose solutions at 0° was about 60% of that in the medium. This was tentatively taken to mean that only 60% of the mitochondrial water was permeable to sucrose.

5. The percentage dry wt. of mitochondrial pellets isolated from 0·25M-sucrose at 0° was 33·9. The presence of polyglucose in the external medium increased this value consistently to 35·3.

6. The concentrations (millimolal) of potassium, orthophosphate and organic phosphate in the internal water of mitochondria isolated from 0·25M-sucrose at 0° were about 50, 7 and 40. The use of other salt-poor isolation and suspending media did not result in higher values for these components.

7. Increase in the concentration of sodium chloride and potassium chloride in the various suspending media in each case by about 0·04 molal caused roughly the same increase in the concentration of these salts in the mitochondrial water. Thus these added substances appear to be passively distributed between mitochondria and medium, and the 'bound' ions, in particular potassium, remain fixed within the mitochondria.

8. The addition of adenine nucleotides and of 2:4-dinitrophenol to the suspending medium at 0° caused changes in the mitochondrial phosphates and potassium.

We wish to thank Professor H. A. Krebs, F.R.S., for his interest and advice in the work. We are especially grateful to Miss B. M. Notton, who contributed many ideas and competent technical assistance. Thanks are also due to Glaxo Ltd. and Bengers Ltd. for the generous gift of dextran, and to Dr P. T. Mora for supplying the polyglucose. One of us (W.C.W.) is a Research Fellow of the National Cancer Institute, U.S. Public Health Service. The work was aided by a grant from the Rockefeller Foundation.

REFERENCES

Appelmans, F., Wattiaux, R. & Duve, C. de (1955). *Biochem. J.* **59**, 438.
Bacon, J. S. D. & Bell, D. J. (1948). *Biochem. J.* **42**, 397.
Bartley, W. (1953). *Biochem. J.* **54**, 677.
Bartley, W. & Davies, R. E. (1954). *Biochem. J.* **57**, 37.
Beaufay, H., Hers, H. G., Berthet, J. & Duve, C. de (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 1539.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Birbeck, M. S. C. & Reid, E. (1956). *Biochim. biophys. Acta*, **20**, 419.
Dalton, A. J. (1957). *Symp. Soc. exp. Biol., Oxford*, 1955 (in the Press).
Fonnesu, A. & Davies, R. E. (1955). *Biochem. J.* **61**, vi.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Hogeboom, G. H. (1955). In *Fine Structure of Cells*, p. 8. *Un. int. Sci. biol.* Ser. B, no. 21. Groningen: P. Noordhoff Ltd.
Isbell, H. S. (1953). *U.S. Pat.* 2 653 931. [*Chem. Abstr.* (1954), **48**, 958h.]
Klemperer, H. G. (1955). Ph.D. Thesis: University of Sheffield.
Kulka, R. G. (1956). *Biochem. J.* **63**, 542.
MacFarlane, M. G. & Spencer, A. G. (1953). *Biochem. J.* **54**, 569.
Mora, P. T. & Pacsu, E. (1955). *U.S. Pat.* 2 719 179.
Novikoff, A. B. (1956). *Proc. 3rd Int. Congr. Biochem., Brussels*, 1955, p. 315.
Pacsu, E. & Mora, P. T. (1950). *J. Amer. chem. Soc.* **72**, 1045.
Price, C. A. & Davies, R. E. (1954). *Biochem. J.* **58**, xvii.
Sanderson, P. H. (1952). *Biochem. J.* **52**, 502.
Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.
Slater, E. C. & Cleland, K. W. (1953). *Biochem. J.* **55**, 566.
Stanbury, S. W. & Mudge, G. H. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 675.
Tedeschi, H. & Harris, D. L. (1955). *Arch. Biochem. Biophys.* **58**, 52.
Whittam, R. & Davies, R. E. (1954). *Biochem. J.* **56**, 445.

Steroids and Other Lipids of Pregnant Goat's Urine

By W. KLYNE and A. A. WRIGHT
Postgraduate Medical School, London, W. 12

(*Received* 31 *October* 1956)

This work was undertaken in association with Dr S. J. Folley, F.R.S., and Dr A. T. Cowie at the National Institute for Research in Dairying, Shinfield, to study the steroids of pregnant goat's urine with particular attention to oestrogens and pregnanediol. It was intended to provide a basis for studies on the metabolism of administered steroids and on pathological conditions. It has also served as a preliminary to similar work on pregnant cow's urine. Preliminary accounts of this work have been given already (Wright & Klyne, 1955; Klyne & Wright, 1956*a*).

Previous work

Little work on the constituents of goat's urine has been described in the literature. Anderson (1916) studied the volatile constituents and isolated *p*-cresol and a non-phenolic ketone, $C_{16}H_{16}O$, which was identical with a substance from cow's urine. Küst & Vogt (1934) showed by biological assay that oestrogen was present in the urine of pregnant goats after the second or third month of pregnancy and then increased steadily in amount until parturition. They could not detect oestrogen in the urine of non-pregnant or male goats. Grant (1948) isolated *p*-ethylphenylsulphuric acid from normal goat's urine and from the urine of ovariectomized goats receiving hexoestrol (3:4-di-*p*-hydroxyphenylhexane) and progesterone. He concluded that this conjugate was quantitatively the most important aryl-sulphuric acid present. Boscott (1950) was unable to isolate any progesterone metabolites from the urine of female goats receiving 100 mg. of progesterone daily. Goto, Oshima & Ugami (1955) used a diphenylamine reagent to estimate 'corticoid' substances in goat's urine.

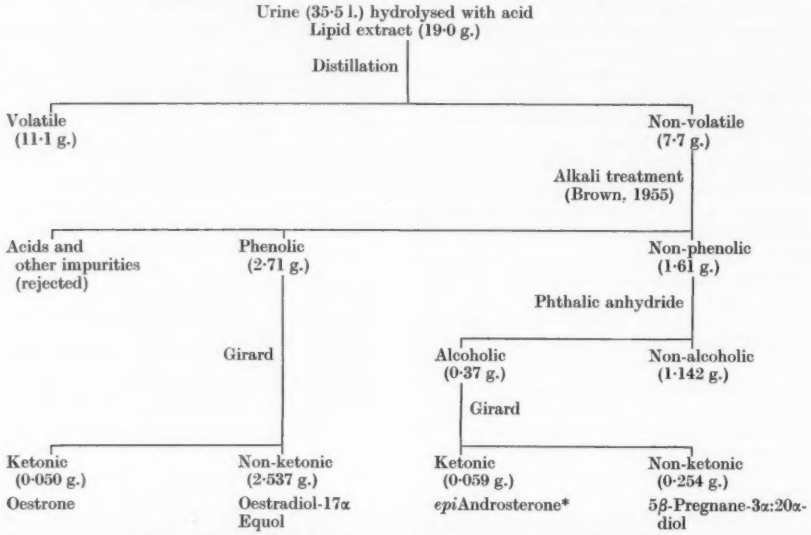
RESULTS

Separation of the major lipid fractions

The urine, which was preserved under toluene, was submitted to acid hydrolysis and extracted with toluene. The extract was distilled *in vacuo* to

Table 1. *Fractionation of lipid extract of pregnant goat's urine*

The compounds isolated are noted under the fraction in which they were found.



* Impure: three other 17-oxo steroids were partially characterized.

remove the volatile constituents, and the non-volatile material was then purified and separated into phenolic and neutral fractions according to the methods of Brown (1955).

The neutral fraction was separated into alcohols and non-alcohols with phthalic anhydride (Dobriner, Lieberman & Rhoads, 1948; cf. Chibnall, 1931). The alcohols were further fractionated by the Girard procedure and digitonin precipitation (Marker, Rohrmann & Wittle, 1938*a*) as used by Brooks, Klyne, Miller & Paterson (1952).

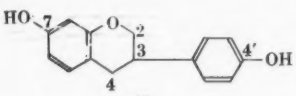
The phenols were also submitted to the Girard procedure. The non-ketonic phenols were then partitioned between water and benzene–light petroleum (Brown, 1955); this separates oestriol, (hydrophilic) from oestradiol (lipophilic). The procedure is summarized in Table 1.

The various major fractions were then submitted to chromatography on alumina, and some fractions were further investigated by paper chromatography and infrared spectrometry.

Phenolic fractions

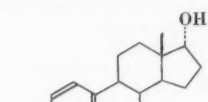
Ketonic phenols. Oestrone was isolated (approx. 0·3 mg./l.) and fully identified. No indication was obtained of the presence of other phenolic ketosteroids.

Non-ketonic phenols. The hydrophilic fraction yielded much equol (*iso*flavan-7:4′-diol, I; Marrian & Haslewood, 1932), but no oestriol. The lipophilic fraction also contained much equol, and a smaller concentration of a Kober chromogen, presumably oestradiol.



(I)

Equol (*iso*flavan-7:4′-diol)



(II)

Oestradiol-17α

Methylation of equol (a dihydric phenol) yields a dimethyl ether, whereas oestradiol yields only a monomethyl ether, which still retains a free alcoholic hydroxyl group. These two methyl ethers could be readily separated by chromatography on alumina, as expected. It was then found that the oestradiol present in pregnant goat's urine was the 17α isomer (II); the concentration of this was about 0·1 mg./l.; none of the 17β epimer could be detected. A modification of the colour reaction of Kägi & Miescher (1939) was used to estimate oestradiol-17α; this modified reaction is also of value for other 17α-hydroxy steroids.

Neutral fractions

Neutral ketones. The only 17-oxo steroid isolated from pregnant goat's urine was *epi*androsterone (3β-hydroxy-5α-androstan-17-one) in an impure condition. Evidence from paper chromatography and infrared spectra indicated the presence of androsterone and aetiocholanolone [3α-hydroxy-5α-(and -5β-)androstan-17-ones] and of 11-oxoaetiocholanolone (3α-hydroxy-5β-androstane-11:17-dione). The total yield of non-volatile, neutral ketones was approx. 1 mg./l.

Neutral non-ketones. Chromatography of the α fraction (not precipitable by digitonin) as free alcohols and as acetates yielded 5β-pregnane-3α:20α-diol (the common 'pregnanediol' of human pregnancy urine) as the sole recognizable compound (approx. 2 mg./l.). Nothing could be isolated from the small β fraction (precipitable by digitonin).

EXPERIMENTAL

Material

The urine (98 l., worked up in two batches of 62·5 and 35·5 l. respectively) was collected from goats at a late stage of pregnancy and stored under toluene. It represented about thirty-five complete 24 hr. collections. The animals were stall-fed on hay and a concentrate of dredge corn, bran, linseed cake, maize, oats, cod-liver oil and minerals. The collections were made while the goats were confined in metabolism crates.

General methods

Methods for melting points (which are corrected), specific rotations, microanalyses, chromatograms on alumina and preparation of acetates and benzoates were as described by Brooks *et al.* (1952). Ultraviolet absorption spectra were determined in ethanol with either a Beckman (DU) or a Unicam (SP. 500) spectrophotometer.

Infrared absorption spectra were measured in a Grubb–Parsons S3A single-beam or Perkin–Elmer double-beam instrument between 1200 and 900 cm.$^{-1}$ (the 'finger-print' region) in CS_2 or in $CHCl_3$.

Chromatography on alumina. The adsorbant was Al_2O_3 for chromatography (Savory and Moore), activity II (Brockmann & Schodder, 1941). The proportions used were lipid (1 g.), Al_2O_3 (30 g.) and eluent (each run, 100 ml.).

Paper chromatography. This was carried out with Savard's (1953) system of light petroleum–propylene glycol at a temperature of 20–25°.

Zimmermann dinitrobenzene reaction for ketones. This was carried out following the method recommended by the Medical Research Council Committee on Clinical Endocrinology (1951).

Methyl ethers. These were prepared with dimethyl sulphate in borate buffer, following the method of

Brown (1955). A third addition of dimethyl sulphate and 20% (w/v) NaOH was sometimes made.

Kober reaction for oestrogens. This was carried out as recommended by Bauld (1954). The quinol–H_2SO_4 reagent for estimating oestradiol-17β was used on fractions containing oestradiol-17α. Standards were always measured with each series of estimations.

The absorption maxima ($\lambda_{max.}$) of the oestradiols and their methyl ethers differed slightly, as follows: oestradiol-17β, 515 mμ; oestradiol-17α, 520 mμ; oestradiol-17β methyl ether, 520 mμ; oestradiol-17α methyl ether, 525 mμ. [Brown (1955) reported 514 and 518 mμ for oestradiol-17β and its methyl ether respectively.] The values of $10^{-3}\epsilon_{max.}$ varied somewhat from one run to another; approximate values were as follows: oestradiol-17β, 35; oestradiol-17α, 40; oestradiol-17β methyl ether, 38; oestradiol-17α methyl ether, 28. [The value of $10^{-3}\epsilon_{max.}$ calculated from the data of Bauld (1954) for oestradiol-17β is 38.] If the Allen (1950) formula was applied to each compound, using the extinctions at 35 mμ either side of the peak in the correction factor, more constant values of $10^{-3}\epsilon$ were obtained, as follows: oestradiol-17β, 22; oestradiol-17β methyl ether, 23; oestradiol-17α, 26·5; oestradiol-17α methyl ether, 22.

When oestradiol-17α was estimated by using oestradiol-17β as standard (the Allen correction being applied as for the 17β isomer) it produced about 10% more colour than an equal weight of the 17β compound. This procedure was followed in the first few determinations before the oestradiol was identified as the 17α isomer. It was continued, for the sake of convenience, in the determination of all fractions which contained any oestradiol.

Equol gave a yellow–brown colour in the Kober reaction, the corrected extinction value at 520 mμ being less than 1% of that for the same weight of oestradiol-17α. (It should be noted that equol may be present in 10- to 20-fold excess in crude fractions obtained after chromatography of free phenols.) Determinations carried out on mixtures of equol and oestradiol showed that the extinctions produced were approximately additive.

Acetic–sulphuric acid reaction

This procedure was based on the reaction used by Kägi & Miescher (1939) to distinguish between 17α- and 17β-hydroxy steroids.

Oestradiol-17α. The acid reagent was prepared by mixing 1 vol. of conc. H_2SO_4 with 20 vol. of acetic acid. This reagent (4 ml.) was added to the dry residue containing 10–40 μg. of oestradiol-17α in a glass-stoppered tube. The mixture was heated by plunging the tube into an oil bath at 130–140° for 2 min. After cooling to room temperature in a bath of cold water, the pink colour ($\lambda_{max.}$, 525 mμ; $10^{-3}\epsilon_{max.}$, 17) was read in a spectrophotometer at this wavelength against a reagent blank. The addition of bromine to the cooled reaction mixture, as recommended by Kägi & Miescher (1939), was found to cause rapid fading of its colour and was therefore omitted.

The absorption spectrum of the pink colour produced by oestradiol-17α resembled that given by the product of the Kober reaction. In applying the method to urinary residues, many of which were brown gums, the colour correction of Allen (1950) was used in order to correct for non-specific absorption. The colour was then read at 490, 525 and 560 mμ.

Concentrations of 2–5 μg. of oestradiol-17α/ml. of the final reaction mixture gave convenient extinction values (0·1–0·3; 1 cm. cell) which were proportional to concentration; typical values for the corrected extinction at 525 mμ were 0·103 and 0·197 for concentrations of 2·25 and 4·5 μg. of oestradiol 17α/ml. respectively.

Other 17α-hydroxy steroids. The absorption spectra of the colours given by some other 17α-hydroxy steroids in this reaction are shown in Fig. 1. 5α-Androstan-17α-ol and 5α-androstane-3β:17α-diol both gave a pink colour resembling that given by oestradiol-17α. The products obtained from the two 5β-androstane-3:17α-diols (aetiocholanediols) were green, but that of androst-5-ene-3β:17α-diol was blue (cf. Kägi & Miescher, 1939). The last-named compound was the only one in this series which gave a colour ($\lambda_{max.}$ 550 mμ) of intensity ($10^{-3}\epsilon_{max.}$ 10·4) comparable with that given by oestradiol-17α.

Oestradiol-17β, 5α-androstane-3β:17β-diol and androst-5-ene-3β:17β-diol gave negligible colours in this reaction.

Equol. Equol was tested at concentrations, 25–50 μg./ml., similar to those present in some of the urinary fractions that were estimated for oestradiol-17α. It gave a green colour, $\lambda_{max.}$ 340, 400 and 597 mμ with $10^{-3}\epsilon_{max.}$ of 1·08, 1·31 and 0·47 respectively. At the wavelengths at which the absorption of oestradiol-17α was normally measured (490, 525, 560 mμ) the spectrum of equol showed a minimum ($10^{-3}\epsilon$ 0·50, 0·39, 0·45 respectively). Determinations of oestradiol-17α in the presence of a 10- to 20-fold excess of equol nevertheless showed a slight increase (5–10%) in the corrected extinction values at 525 mμ as compared with those for pure oestradiol-17α.

Hydrolysis and extraction of urine

The urine was received with some toluene added as a preservative. It was hydrolysed by adding 0·1 vol. of 10 N-HCl and heating in a boiling-water bath for 1·5 hr. in the presence of toluene. The hydrolysate was brought to pH 6 with solid Na_2CO_3 and filtered. More toluene was added to a total of 0·2 vol. and the mixture shaken. The aqueous phase was further extracted with toluene (2 × 0·2 vol., 1 × 0·1 vol.). Precipitates, filtered off after hydrolysis or

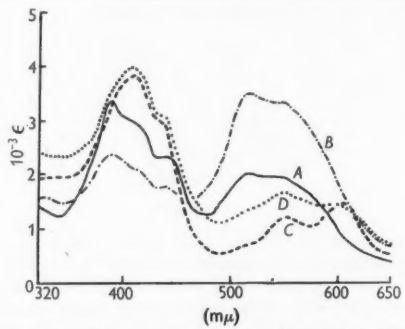


Fig. 1. Absorption spectra of products obtained in acetic–sulphuric acid reaction with some 17α-hydroxy steroids. *A*, 5α-Androstan-17α-ol; *B*, 5α-androstane-3β:17α-diol; *C*, 5β-androstane-3α:17α-diol; *D*, 5β-androstane-3β:17α-diol.

from the emulsions formed during extraction, were also extracted with toluene, which was then added to the bulk. The toluene extract was washed with water (4 × 0·05 vol.) and evaporated *in vacuo*.

Separation of the major fractions

The lipid material obtained (22·25 g. from 62·5 l. of urine) was distilled at 120°/10–15 mm. for 1 hr., and then for a further 1 hr. at 120°/1 mm., giving two volatile fractions (V_1, 4·27 g., and V_2, 5·4 g., respectively).

The non-volatile residue, 12·36 g., was dissolved in ether (4 l.) and acidic or oxidizable constituents were removed by the method of Brown (1955) as follows. (*A*) The ethereal solution was first washed with 2 × 800 ml., 2 × 400 ml. of conc. carbonate solution [prepared by adding NaOH (20%, w/v; 150 ml.) to $NaHCO_3$ (8%, w/v, 1 l.)]; these washings were rejected. (*B*) The ethereal layer was next shaken with 2N-NaOH (200 ml.); $NaHCO_3$ (8%, w/v; 800 ml.) was then added and the mixture shaken again before the layers were separated; the washing was rejected. (*C*) Stage *B* was repeated with half the volumes of NaOH and $NaHCO_3$. (*D*) and (*E*) The ethereal solution was finally washed with $NaHCO_3$ (8%, w/v; 400 ml.) and water (4 × 100 ml.), all washings being rejected. The residue after evaporation of the ether (6·68 g.) was dissolved in benzene (1 l.) and the phenols were extracted by washing with 2N-NaOH (1 × 100 ml., 7 × 50 ml.). The benzene was then washed with water (3 × 25 ml.), dried over Na_2SO_4 and evaporated, giving a neutral fraction (2·647 g.). The NaOH solutions were brought to pH 2·0 with 10N-HCl and saturated with NaCl. They were then extracted with ether (3 × 100, 3 × 50 ml.). After drying over Na_2SO_4 the ether was evaporated to give the phenolic fraction (2·435 g.).

Phenols. These were separated into ketonic and non-ketonic fractions by the Girard procedure (cf. Brooks *et al.* 1952).

Non-phenolic fraction. This was treated with phthalic anhydride (Dobriner *et al.* 1948) to obtain alcoholic and non-alcoholic fractions. The alcohols were further fractionated to obtain ketonic and non-ketonic alcohols (Table 1), each of which gave digitonin-precipitable and -non-precipitable fractions (Brooks *et al.* 1952). The digitonin precipitations were carried out in 95% ethanol as described by Marker *et al.* (1938*a*). As only a slight cloudiness was obtained after the ethanol solutions had stood for 3 hr., the solutions were concentrated and precipitated with ether. The β fractions were then contained wholly in the ether-precipitated material.

Phenolic fractions

Ketonic phenols

After the Girard separation, 85 mg. of ketonic material was obtained from 62·5 l. of urine. It was chromatographed on alumina (2·7 g.) and the following fractions were obtained: chromatogram I *A* (benzene), 4 mg.; *B* (runs 4–8; benzene–ether, 90:10), 27 mg.; *C* (benzene–ether, 80:20) 6 mg.; *D* (benzene–ether, 50:50, ether), traces; *E* (ether–methanol, 50:50), 22 mg. Fraction *E* was methylated and chromatographed again, but no compounds were isolated.

Isolation of oestrone. The first part of fraction I *B* (run 4) was crystalline (16 mg.), and was therefore chromatographed again (chromatogram II). Benzene eluted only traces of gum, but benzene containing 1% of ether-eluted material which crystallized spontaneously. Higher concentrations of ether gave fractions containing only small amounts of gum. The solid fractions were combined and recrystallized from ethyl acetate. The product had m.p. 268–269°, not depressed on admixture with authentic oestrone. A comparison of the properties of this compound and its derivatives with those of authentic material is given in Table 2.

Non-ketonic phenols

The non-ketonic fraction (2·159 g. from 62·5 l. of urine) was dissolved in ethanol (40 ml.) and transferred to a separating funnel with benzene (1 l.). Light petroleum (1 l.) was added and the organic phase extracted with water (2 × 1 l.) (cf. Brown, 1955).

Hydrophilic fraction. The aqueous solutions were allowed to stand two days at 4°, when pale-buff crystals were deposited. These were collected (fraction *A*). One-tenth of the supernatant was methylated (fraction *B*) and the remainder acidified and extracted with ether (fraction *C*).

Equol. The crystals (*A*, 0·357 g.) had m.p. 150–155°. They were apparently insoluble in saturated $NaHCO_3$ and 2N-Na_2CO_3, but dissolved readily in 2N-NaOH and methanol. After recrystallization from water they had m.p. 187–190°, and showed no depression on mixing with authentic equol (Marrian & Haslewood, 1932). Table 3 shows a comparison between the equol isolated in this work and authentic material. The product of the methylation (*B*, 0·057 g.) was chromatographed on alumina (chromatogram III). Light petroleum–benzene (95:5) eluted 43 mg. of material which was identified as equol dimethyl ether (cf. Table 3). Further elution with light petroleum–benzene, benzene and benzene–ether gave no identifiable material. More impure equol was obtained by chromatography of fraction *C*. This fraction contained only a small amount of Kober chromogen, and nothing else was obtained from it.

Table 2. *Identification of oestrone*

Phenol	Urinary compound		Authentic oestrone
M.p.	268–269°		266–267°
Mixed m.p.		270–271°	
Ultraviolet absorption ($\lambda_{max.}$)	280 mμ	Identical	280 mμ
Infrared spectrum (KBr disk)			
Kober reaction ($\lambda_{max.}$)	515 mμ		515 mμ
Acetate			
Infrared spectrum (CS_2)		Identical	

Lipophilic non-ketonic phenols

The phenols remaining in the organic phase at the partition (1·284 g.) were chromatographed on alumina (chromatogram IV; Table 4). Kober determinations indicated that the oestrogen was mainly in fraction *C* and the adjacent eluates in fractions *B* and *D*; a smaller amount was present in fraction *G*.

Oestradiol-17α. The oestrogen was only a small proportion of each fraction. The predominant material appeared from its behaviour on chromatography and its solubility in various solvents to be equol. After methylation of the crude product, equol dimethyl ether and oestradiol monomethyl ether could be separated readily by chromatography (Table 4).

Fraction V*A* on recrystallization from methanol yielded crystals, m.p. 88·5–90°, not depressed on admixture with authentic equol dimethyl ether. Fraction V*D* was recrystallized from pentane–ether, yielding crystals, m.p. 111–112·5°, not depressed by admixture with an authentic sample of oestradiol-17α methyl ether. This material was further investigated by means of the Kober and acetic-

Table 3. *Identification of equol*

Phenol	Urinary compound		Authentic equol
M.p.	187–190°		190–192°
Mixed m.p.		190–192°	
Infrared spectrum ($CHCl_3$)		Identical	
$[\alpha]_D$ (EtOH)	−17°		−22°*
Methyl ether			
M.p.	86·5–87·5°		88·5–89·5°
Mixed m.p.		88–89·5°	
Infrared spectrum (CS_2)		Identical	
Diacetate			
M.p.	123–125°		122–124°
Mixed m.p.		123–125°	
Infrared spectrum (CS_2)		Identical	
Dibenzoate			
M.p.	187–190°; 219–220·5°		189·5–190·5°; 209–212°
Mixed m.p.		188–190°; 216–219·5°	
Infrared spectrum (CS_2)		Identical	

* Marrian & Haslewood (1932) found $[\alpha]_{5461}$ −21·5° (EtOH).

Table 4. *Chromatography of lipophilic phenols as free phenols and as methyl ethers*

Tube no.	Eluent	Eluate: Fraction	Eluate: Wt. (mg.)	Eluate: Appearance	Kober chromogen
	Chromatogram IV: free phenols. 40 g. of Al_2O_3; each run 120 ml. of eluent				
1–4	Benzene	IV*A*	44	Gum	−
5–6	Benzene–ether (90:10)	IV*B* (1)	2	Gum	−
7–9	Benzene–ether (90:10)	IV*B* (2)	37	Gum	+ +
10–13	Benzene–ether (80:20)	IV*C*	56	Solid	+ + +
14–15	Benzene–ether (60:40)	IV*D* (1)	42	Solid	+ +
16–21	Benzene–ether (60:40)	IV*D* (2)	59	Solid	tr.*
22–23	Benzene–ether (40:60)	IV*E*	20	Solid	tr.
24–26	Ether	IV*F*	18	Solid	tr.
27–30	Ether–methanol (90:10)	IV*G*	329	Gum	+ +†
31–37	Ether–methanol (90:10; 50:50)	IV*H*	—	Gum	tr.
	Chromatogram V: methyl ethers. Fractions IV *B* (2), *C*, *D* (1) after methylation. 3 g. of Al_2O_3; each run 10 ml. of eluent				
1–6	Light petroleum–benzene (95:5)	V*A*	49	Solid	−
7–12	Light petroleum–benzene (90:10; 80:20)	V*B*	.	Gum	−
13–16	Light petroleum–benzene (50:50)	V*C*	.	Gum	+ +
17	Benzene	V*D*	7	Solid	+ + +
18–20	Benzene	V*E*	.	Gum	+ +
21–22	Benzene–ether (90:10)	V*F*	.	Gum	tr.
23–26	Benzene–ether (80:20); ether	V*G*	.	Gum	−

* tr. = trace. † This fraction contained much equol; see text, chromatogram VI.

Table 5. *Identification of oestradiol-17α*

	Urinary compound		Authentic oestradiol-17α
3-Methyl ether			
M.p.	111–112·5°		113–114°
Mixed m.p.		112–114°	
Infrared spectrum (CS_2)		Identical	
Kober reaction, $\lambda_{max.}$	526 mμ		525 mμ
Acetic–sulphuric acid reaction, $\lambda_{max.}$	523 mμ		524 mμ
17α-Acetate 3-methyl ether			
M.p.	126·5–129°		129–130°
Mixed m.p.		128·5–130·5°	
Infrared spectrum (CS_2)		Identical	

Oestradiol-17α-acetate 3-methyl ether, authentic (Found: C, 76·7; H, 8·5. $C_{21}H_{28}O_3$ requires C, 76·8; H, 8·6%).

Table 6. *Chromatography of the α-ketonic alcohols*

Chromatogram VII: α-ketonic alcohols, 87 mg.; Al_2O_3, 2·7 g.; each run 9 ml. of solvent. 'Ketosteroids' mean substances giving Zimmermann-positive spots with R_F of the same value as the known compounds indicated. An, androsterone; Ae, aetiocholanolone; K, 11-oxoaetiocholanolone. Figures, thus (+2), show the number of additional Zimmermann-positive spots of unknown origin. tr. = trace.

		Eluate		
Tube no.	Eluent	Fraction	Weight (mg.)	'Ketosteroids' present
1	Light petroleum–benzene (25:75)	VII*A*	2	—
2	Light petroleum–benzene (25:75)	VII*B*	12	An
3	Light petroleum–benzene (25:75)	VII*C*	7	An, Ae, +2
4–6	Benzene	VII*D*	10	An (faint), Ae, +4
7–10	Benzene	VII*E*	6	+2
11–14	Benzene–ether (90:10)	VII*F*	7	Ae, K, +2
15–17	Benzene–ether (80:20)	VII*G*	tr.	K, +1
18–20	Benzene–ether (50:50)	VII*H*	tr.	K, +1
21–23	Ether	VII*J*	tr.	K (faint), +1
24–26	Ether–methanol (90:10)	VII*K*	tr.	K, +1
27–29	Ether-methanol (50:50)	VII*L*	—	—

sulphuric acid reactions. To provide an additional comparison, samples of oestradiol-17α 17-acetate 3-methyl ether were prepared. Comparisons of the compound from goat's urine with authentic material are shown in Table 5.

Fraction IV*G* was also methylated and chromatographed on alumina (chromatogram VI); fraction VI*A* (light petroleum–benzene, 90:10 and 80:20) contained 229 mg. of material which proved to be impure equol dimethyl ether. No other crystalline material was obtained. Fractions VI*D*, *E* and *F* (eluted with various benzene–ether mixtures) contained small amounts of Kober chromogens (see next paragraph).

Possible occurrence of oestradiol-17β. Kober and acetic–sulphuric acid estimations were carried out on all fractions of the above chromatograms that might contain oestradiol. Values for total oestradiol (Kober) and oestradiol-17α (acetic–sulphuric) agreed to within 10% for all fractions except VI*D*, *E* and *F*. Interfering substances prevented accurate evaluation of the proportions of 17α and 17β isomers in these three fractions. Calculation from the differences between the two series of results as a whole indicated that oestradiol-17β could not have represented more than 10% of the total oestradiol present; in fact there was no positive evidence for the presence of the 17β isomer.

Non-phenolic fractions

α-Ketonic alcohols

The ketonic alcohols not precipitated by digitonin (87 mg.) were chromatographed on alumina (chromatogram VII). The eluates were examined by paper chromatography with the light petroleum–propylene glycol system of Savard (1953). The results are shown in Table 6. Spots were obtained with the same R_F values as androsterone, aetiocholanolone and 11-oxoaetiocholanolone. Five other spots were obtained with the Zimmermann reagent, the colours ranging from blue to brown.

Some selected fractions of this main chromatogram were combined and rechromatographed on alumina (chromatogram VIII). Examination by paper chromatography (Savard, 1953) and infrared spectrometry of the fractions so obtained indicated that they were still complex mixtures. The infrared spectra indicated the presence of aetiocholanolone and 11-oxoaetiocholanolone by reason of similarities with authentic spectra in the regions near 1036 cm.$^{-1}$ and 1000–1200 cm.$^{-1}$ respectively. Many fractions also showed a peak at or about 1670 cm.$^{-1}$ typical of a 4-en-3-one grouping. We are indebted to Dr A. E. Kellie of the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, for measuring and interpreting these infrared spectra.

Fraction VII*C* was submitted to gradient-elution chromatography (Kellie & Wade, 1956) by Dr A. E. Kellie. He reported that the 17-oxo steroids were separated from one another (as in samples from human urine) but that they could not be separated from the other substances present, and no quantitative estimation could therefore be carried out. Paper chromatography with the system *A* of Bush (1950) confirmed our finding of etiocholanolone and androsterone; many other substances were also found.

β-Ketonic alcohols

The ketonic alcohols precipitated by digitonin (21 mg.) were also chromatographed on alumina (chromatogram IX) and the fractions were investigated by paper chromatography. When the Zimmermann reagent was used for identification, only two spots could be obtained. One of these, eluted from the alumina with ether–methanol mixtures, was faint. The other substance was eluted from alumina with benzene–ether (90:10) and appeared from its R_F value to be *epi*androsterone. The relevant fractions were combined and chromatographed again on alumina (chromatogram X). Material eluted with benzene and benzene–ether mixtures (95:5) partly solidified on treatment with ether and methanol. The most solid fraction was recrystallized from ethyl acetate–pentane. Crystals were obtained which melted at 173–176° after considerable previous softening; there was insufficient material for a mixed-melting-point determination with authentic material (m.p. 174–178°). The infrared spectra of the crystals showed a clear pattern of *epi*androsterone, but indicated the presence of impurities, including some 20-oxo steroid as shown by the typical peak (1705 cm.$^{-1}$). Dr Kellie estimated that the material contained approx. 70% of *epi*androsterone. No dehydro*epi*androsterone could be detected on the paper chromatograms, but the colour reaction of Allen, Hayward & Pinto (1950) indicated that a trace might be present.

α-Non-ketonic alcohols

This fraction was chromatographed on alumina (12 g.; chromatogram XI); the following fractions (all gums) were eluted with the solvents indicated: *A*, benzene, 37 mg.; *B*, *C* and *D*, benzene–ether mixtures, 33, 42 and 54 mg.; *E*, ether, 17 mg.; *F*, ether–methanol (90:10), 176 mg.; *G*, ether–methanol (80:20 and 50:50), 19 mg. Fraction XI*F* was immediately chromatographed again (chromatogram XII, Table 7). Fractions XI*E* and XII*B*, which showed signs of solidification, were combined and chromatographed (chromatogram XIII). Finally, fractions XII*C* and XII*D* were also chromatographed again (chromatograms XIV, XV respectively). Many fractions eluted with ether and with ether–methanol (99:1, 98:2) solidified. Attempts were made to recrystallize these from ether and then methanol, but the melting points of the fractions varied from 200° to 230°. The best fractions were therefore acetylated separately, and the products recrystallized from methanol. All of these had m.p. 164–168°, and on admixture with authentic 5β-pregnane-3α:20α-diol diacetate the m.p. showed no depression (Table 8).

Table 7. *Chromatography of the α-non-ketonic alcohols*

Chromatogram XII: fraction XI*F*. Al_2O_3, 10 g.; each run 20 ml. of solvent.

		Eluate			
Tube no.	Eluent	Fraction	Wt. (mg.)	Appearance	M.p.
1–6	Benzene–ether (50:50; 20:80)	XII*A*	8	Gum	—
7–13	Ether	XII*B*	12	Gum and solid	—
14	Ether–methanol (98:2)	XII*C*	47	Gum and solid	—
15–16	Ether–methanol (98:2)	XII*D*	57	Gum	—
17	Ether–methanol (98:2)	XII*E*	3	Gum and solid	230–231·5°*
18	Ether–methanol (98:2)	XII*F*	5	Solid	200–210°
19	Ether–methanol (98:2)	XII*G*	3	Gum and solid	215–218°*
20	Ether–methanol (95:5)	XII*H*	5	Solid	224–227°*
21–32	Ether–methanol (95:5; 90:10; 80:20; 50:50)	XII*J*	15	Gum	—

* M.p. after crystallization.

Table 8. *Identification of 5β-pregnane-3α:20α-diol*

	Urinary compound		Authentic 5β-pregnane-3α:20α-diol
Diol			
M.p.	238·5–239°		236·5–239°
Mixed m.p.		241–243°*	
Diacetate			
M.p.	167–168°		168–169°
Mixed m.p.		167–168·5°	
Infrared spectrum (CS_2)		Identical	
Dibenzoate			
Infrared spectrum (CS_2)		Identical	

* A similar rise was found when the mixed melting point was repeated. No explanation can be given.

5β-Pregnane-3α:20α-diol. The acetates prepared as described above were combined and hydrolysed with KOH (5%, w/v) in aqueous methanol under reflux for 2 hr. The reaction product was dissolved in ether (25 ml.) and washed with water (1 × 4 ml., 4 × 2 ml.). The ether was evaporated and the residue recrystallized from methanol. The product was compared with authentic 5β-pregnane-3α:20α-diol (see Table 8). Finally the benzoate was prepared for a comparison of the infrared spectrum with that of authentic material.

β-Non-ketonic alcohols

This fraction was small (18 mg.). After chromatography on alumina, the fraction eluted with ether–methanol (95:5 and 90:10) was the largest; this was chromatographed again. Nothing was identified, even after acetylation and chromatography of the acetates on alumina.

DISCUSSION

The results of this work emphasize two points which are also obvious from previous work on species differences in urinary steroids (cf., for example, Brooks & Klyne, 1957), but which have unfortunately been disregarded in some other studies: first, that a thorough qualitative investigation of the urinary steroids in any species should be made before their estimation is undertaken; secondly, that care must be taken in applying to animal urines, which may contain unknown interfering constituents, methods designed for human urine. The pregnant goat excretes only small quantities of steroids, whose estimation may be considerably hampered by other materials present.

Pregnanediol

We have isolated 5β-pregnane-3α:20α-diol from pregnant goat's urine; the presence of other isomers could not be demonstrated. 5β-Pregnane-3α:20α-diol is the common 'pregnanediol' of human pregnancy urine, which contains up to 50 mg./day or more in late pregnancy; the goat excretes a much smaller quantity (approx. 5 mg./day). Meites, Webster, Young, Thorp & Hatch (1951) found that pregnant goats from which corpora lutea had been removed required about 10 mg. of progesterone/day to maintain pregnancy. This has been confirmed by Amoroso (unpublished observations).

The pregnant mare excretes a mixture of diols (Brooks *et al.* 1952; Brooks & Klyne, 1957). 5β-Pregnane-3α:20α-diol has also been demonstrated in the pregnancy urine of the chimpanzee (Fish, Dorfman & Young, 1942) and the rabbit (Verly, Sommerville & Marrian, 1950). In the latter animal it was identified by Heard, Bauld & Hoffman (1941) as the principal metabolite of progesterone. Marker (1938) and Marker, Wittle & Lawson (1938*b*) claimed that 5β-pregnane-3α:20α-diol was the main diol component of the neutral steroids of bull's and cow's urine; Klyne & Wright (1956*b*) were, however, unable to find any pregnanediol in pregnant cow's urine.

Oestrogens

The chief C_{18} steroid in pregnant goat's urine is oestrone (about 0·3 mg./l. or 0·8 mg./24 hr.); a smaller quantity (about 0·1 mg./l.) of oestradiol-17α has been isolated. The application of the Kober reaction and the acetic–sulphuric acid reaction to all the urinary fractions containing oestradiol indicated that little or none of the 17β-isomer was present.

Oestrone is the major phenolic steroid excreted by the mare at mid-pregnancy [up to 200 mg./l. (Beall & Grant, personal communication); 30 mg. of oestrogen (as oestrone)/l. (Kober, 1938)], but in late pregnancy the *B*-ring unsaturated ketones (equilin and equilenin) predominate. Some confusion exists about the proportions of the non-ketonic phenols of mare's urine. The work of Grant and his colleagues (Beall & Grant, personal communication; Glen, Barber, McConkey & Grant, 1956) indicates that 3:17α-diols [oestradiol-17α, dihydroequilin-17α and dihydroequilenin-17α (previously called 'β'-oestradiol, 'β'-dihydroequilin and 'β'-dihydroequilenin respectively)] are the major constituents of the non-ketonic phenolic fraction; the proportions of the compounds differ at different stages of pregnancy. Earlier workers (Hirschmann & Wintersteiner, 1938; Stolk & Lenchère, 1937) had found that oestradiol-17β could be isolated in larger amounts than the 17α-isomer.

The rabbit excretes oestradiol-17α after injection with oestrone (Stroud, 1939; Pearlman & Pearlman, 1944) or with oestradiol-17β (Heard *et al.* 1941; Fish & Dorfman, 1941); oestrone, the only other product, is excreted in smaller amounts. Pearlman & Pearlman (1944) found no evidence for the presence of oestradiol-17α in human pregnancy urine.

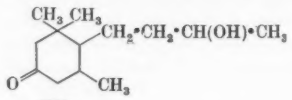
Ketosteroids

The ketosteroids of goat's urine have not been completely characterized in this work, mainly because the quantities are very small. The concentrations of the individual 17-oxosteroids have not been estimated, but the total non-volatile ketonic-alcohol fraction amounts to little more than 1 mg./l. This is similar to the value estimated by Holtz (1956) in cattle urines (0·3 mg. of 17-oxo steroids/l.).

Ionone derivatives

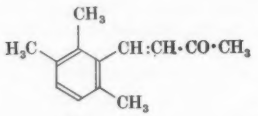
The estimation of 17-oxo steroids in pregnant goat's urine by the routine Zimmermann procedure (M.R.C. Committee on Clinical Endocrinology, 1951) gives values very much higher than the quantities isolated. 'Apparent 17-ketosteroid'

values for individual 24 hr. specimens were 7–10 mg./l., or 10–23 mg./24 hr. Holtz (1954, 1956) has investigated the application of this method to cattle urines, and has produced evidence for the presence of a number of non-steroid substances which interfere. These compounds, related to the ionones (e.g. III), have been isolated by Prelog and his coworkers from mare's urine (Prelog, Führer, Hagenbach & Schneider, 1948). One of the volatile ketonic fractions obtained by us from pregnant goat's urine gave a Zimmermann product, the absorption curve of which was similar to those given by a 17-oxo steroid and by the ketone *J* of Prelog *et al.* (1948) (2:3:6-trimethylbenzylideneacetone, IV).



(III)

1-(3-Hydroxybutyl)-2:2:6-trimethyl*cyclo*hexan-4-one
'Oxyketone *E*'



(IV)

2:3:6-Trimethylbenzylideneacetone
'Ketone *J*'

The origin of these compounds is unknown, but it has been suggested that they might be breakdown products of carotenoids (Prelog *et al.* 1948; Holtz, 1954, 1956). It would be interesting to compare the output of 'apparent ketosteroids' by animals on diets of different carotenoid content.

A recent paper by Simon, Eisengart, Sundheim & Milhorat (1956) describes two C_{16} metabolites of α-tocopherol, which must have arisen by fission of a long polyisoprene side chain in a manner similar to that by which the C_{13} ionone derivatives might be derived from carotenoids.

Equol

Many fodder plants contain oestrogenic substances, some of which are *iso*flavones, e.g. formononetin and genistein. This subject has been reviewed by Bradbury & White (1954). Bassett, Sewell & White (1954), using a biological assay, found that sheep grazing on oestrogen-potent red clover excreted, in the urine, appreciably more oestrogen than grass-fed animals. Equol (I), which has been isolated from goat's urine (10–15 mg./l.) in this work, is *iso*flavan-7:4′-diol (Marrian & Haslewood, 1932; Marrian & Beall, 1935; Anderson & Marrian, 1939), and could arise by reduction of the 2:3 double bond and the 4-oxo group of an *iso*flavone. Equol itself is inactive oestrogenically (Marrian & Haslewood, 1932) and would not interfere with biological assay. It might, nevertheless, interfere with chemical estimation; pilot experiments reported on p. 94 indicate that in the Kober reaction the errors introduced by the equol present in goat's urine would probably not exceed about 10%. The effect of equol on the chromatographic separations used in recent chemical estimations of oestrogen (Brown, 1955; Bauld, 1956) has not yet been studied.

SUMMARY

1. Pregnant goat's urine was hydrolysed with acid, and the lipid material obtained was submitted to the fractionation procedures customary in the study of urinary steroids.

2. Oestrone (0·3 mg./l.) and oestradiol-17α (0·1 mg./l.) were isolated from the phenolic fraction. Oestradiol-17β could not be detected.

3. The heterocyclic phenol equol (*iso*flavan-7:4′-diol) was also isolated (10–15 mg./l.).

4. 5β-Pregnane-3α:20α-diol (2 mg./l.), the common 'pregnanediol' of human pregnancy urine, was isolated from the neutral non-ketonic fraction. There was no evidence for the presence of any isomeric pregnanediols.

5. The neutral non-volatile ketonic fraction was small (1 mg./l.). *epi*Androsterone (3β-hydroxy-5α-androstan-17-one) was isolated from it in impure form. Three other α-17-oxo steroids were partially characterized.

6. Much other volatile material giving a purple colour in the Zimmermann dinitrobenzene reaction was present. This 'apparent 17-ketosteroid' material probably includes ionone derivatives (cf. Holtz, 1954).

7. Species differences in urinary steroids are discussed.

We are indebted to the Agricultural Research Council for a grant for expenses which has largely defrayed the costs of this work, and for a personal grant to one of us (A. A. W.). We are most grateful to Dr S. J. Folley, F.R.S., and Dr A. T. Cowie (National Institute for Research in Dairying, Shinfield) for supplies of pregnant goat's urine and for helpful discussions; to Dr A. E. Kellie (Courtauld Institute of Biochemistry, Middlesex Hospital) and to Dr I. D. P. Wootton and Miss Sylvia Saunders (this Department) for recording and interpreting infrared spectra; to Miss Miriam Mattock for '17-ketosteroid' determinations; and to the following for gifts of materials; Professor G. F. Marrian, F.R.S. (Edinburgh; equol), Organon Laboratories (oestradiol-17α), Professor C. W. Shoppee, F.R.S. (Swansea; 5α-androstan-17α-ol), Dr R. K. Callow (National Institute for Medical Research; 5β-androstane-3α:17α-diol) and Mr P. M. Jones (5α- and 5β-androstane-3β:17α-diols).

REFERENCES

Allen, W. M. (1950). *J. clin. Endocrin.* **10**, 71.
Allen, W. M., Hayward, S. J. & Pinto, A. (1950). *J. clin. Endocrin.* **10**, 54.
Anderson, E. L. & Marrian, G. F. (1939). *J. biol. Chem.* **127**, 649.
Anderson, R. J. (1916). *J. biol. Chem.* **26**, 401.
Bassett, E. G., Sewell, O. K. & White, E. P. (1954). *N.Z. J. Sci. Tech.* A, **36**, 437.
Bauld, W. S. (1954). *Biochem. J.* **56**, 426.
Bauld, W. S. (1956). *Biochem. J.* **63**, 488.
Boscott, R. J. (1950). *Ciba Foundation Colloq. Endocrin.* **2**, 327.
Bradbury, R. B. & White, D. E. (1954). *Vitam. & Horm.* **12**, 207.
Brockmann, H. & Schodder, H. (1941). *Ber. dtsch. chem. Ges.* **74**, 73.
Brooks, R. V. & Klyne, W. (1957). *Biochem. J.* **65**, 663.
Brooks, R. V., Klyne, W., Miller, E. & Paterson, J. Y. F. (1952). *Biochem. J.* **51**, 694.
Brown, J. B. (1955). *Biochem. J.* **60**, 185.
Bush, I. E. (1950). *Biochem. J.* **50**, 370.
Chibnall, A. C. (1931). *Biochem. J.* **25**, 2108.
Dobriner, K., Lieberman, S. & Rhoads, C. P. (1948). *J. biol. Chem.* **172**, 241.
Fish, W. R. & Dorfman, R. I. (1941). *J. biol. Chem.* **140**, xl.
Fish, W. R., Dorfman, R. I. & Young, W. C. (1942). *J. biol. Chem.* **143**, 715.
Glen, W. L., Barber, R., McConkey, H. M. & Grant, G. A. (1956). *Nature, Lond.*, **177**, 753.
Goto, T., Oshima, M. & Ugami, S. (1955). *Bull. nat. Inst. agric. Sci. Japan*, G, **10**, 89.
Grant, J. K. (1948). *Biochem. J.* **43**, 523.
Heard, R. D. H., Bauld, W. S. & Hoffman, M. M. (1941). *J. biol. Chem.* **141**, 709.
Hirschmann, H. & Wintersteiner, O. (1938). *J. biol. Chem.* **122**, 303.
Holtz, A. H. (1954). *Nature, Lond.*, **174**, 316.
Holtz, A. H. (1956). Thesis: University of Utrecht.
Kägi, H. & Miescher, K. (1939). *Helv. chim. acta*, **22**, 683.
Kellie, A. E. & Wade, A. P. (1956). *Biochem. J.* **62**, 1P.
Klyne, W. & Wright, A. A. (1956*a*). *Biochem. J.* **62**, 21P.
Klyne, W. & Wright, A. A. (1956*b*). *J. Endocrin.* **14**, xxxiii.
Kober, S. (1938). *Arch. int. Pharmacodyn.* **59**, 61.
Küst & Vogt (1934). *Tierärztl. Rdsch.* **40**, 589.
Marker, R. E. (1938). *J. Amer. chem. Soc.* **60**, 2442.
Marker, R. E., Rohrmann, E. & Wittle, E. L. (1938*a*). *J. Amer. chem. Soc.* **60**, 1561.
Marker, R. E., Wittle, E. L. & Lawson, E. J. (1938*b*). *J. Amer. chem. Soc.* **60**, 2931.
Marrian, G. F. & Beall, D. (1935). *Biochem. J.* **29**, 1586.
Marrian, G. F. & Haslewood, G. A. D. (1932). *Biochem. J.* **26**, 1227.
Medical Research Council Committee on Clinical Endocrinology (1951). *Lancet*, **2**, 585.
Meites, J., Webster, H. D., Young, F. W., Thorp, F. jun. & Hatch, R. N. (1951). *J. Anim. Sci.* **10**, 411.
Pearlman, W. H. & Pearlman, M. R. J. (1944). *Arch. Biochem.* **4**, 97.
Prelog, V., Führer, J., Hagenbach, R. & Schneider, R. (1948). *Helv. chim. acta*, **31**, 1799.
Savard, K. (1953). *J. biol. Chem.* **202**, 457.
Simon, E. J., Eisengart, A., Sundheim, L. & Milhorat, A. T. (1956). *J. biol. Chem.* **221**, 807.
Stolk, D. van & Lenchère, R. L. de (1937). *C.R. Acad. Sci., Paris*, **24**, 249.
Stroud, S. W. (1939). *J. Endocrin.* **1**, 201.
Verly, W. G., Sommerville, I. F. & Marrian, G. F. (1950). *Biochem. J.* **46**, 186.
Wright, A. A. & Klyne, W. (1955). *Abstr. 3rd Int. Congr. Biochem., Brussels*, p. 139.

Protein Synthesis in Ripening Pea Seeds

1. ANALYSIS OF WHOLE SEEDS

By ILSE DOROTHEA RAACKE*

Biochemical Institute, University of Uppsala, Sweden

(*Received* 22 *June* 1956)

Under conditions conducive to protein synthesis, the amount of protein nitrogen increases at the expense of soluble nitrogen; this has been shown by a great number of workers to hold true for developing seeds (cf. Chibnall, 1939; Schulze, 1911; Schulze & Winterstein, 1910; Emmerling, 1880, 1887, 1900; Zaleski, 1905; Nedokutschajew, 1902, 1904; Boswell, 1924; Bisson & Jones, 1932), for leaves (Wood & Petrie, 1942) and for actively metabolizing potato disks (Steward & Preston, 1940, 1941). This fact, however, does not provide any decisive information about the path of protein synthesis, and can be interpreted in the light of several current theories. Wood & Petrie (1942), for example, considered the correlation between amino acid and protein nitrogen as strong evidence in favour of the hypothesis of direct condensation of amino acids, whereas Steward & Preston (1940, 1941), from their studies with metabolizing potato disks, thought that the amino acids were not utilized directly in protein synthesis, but were first deaminated.

It is not possible, at present, to decide between the two hypotheses of protein synthesis mentioned

* Present address: Chemical Laboratory, University of Cambridge.

above; and although that of the direct utilization of amino acids seems to be preferred, there is not sufficient evidence to decide whether the condensation of the amino acids takes place in a single step, i.e. by a 'template' mechanism, or whether it takes place in a stepwise manner. If the latter is true, polypeptide precursors ought to be detectable.

The presence in plants of polypeptides and other relatively low-molecular components termed 'peptones', 'proteoses', or 'albuminoses' has been vaguely inferred by many workers, and some attempts have been made to include the last-named in the analysis of seeds. Nedokutschajew (1902, 1904) determined 'albuminose' in wheat seeds at different stages of development by precipitation with zinc sulphate, and found that the amount of albuminose decreased up to a certain point in the ripening seeds and increased again in the mature grain. Zaleski (1905) determined albuminoses in unripe peas by determining the difference between the nitrogen precipitated by zinc chloride and that precipitated by cupric hydroxide. He found that upon storage of the seeds the amount of albuminoses remained constant, but the amount of protein nitrogen increased by an amount equivalent to that of the albuminoses originally present. From this Zaleski concluded that protein was formed at the expense of the albuminoses and these latter were replenished from materials not precipitated by zinc chloride.

More specific data are required to determine whether these low-molecular substances are involved in the synthesis of proteins and it is the object of the present investigation to supply these.

No one has demonstrated peptides as intermediates in protein synthesis in animal tissues, possibly because of their rapid rate of metabolism. In animals there is also a continuous turnover of protein, and one has to take into consideration the outgoing as well as the incoming nitrogen. Plants, on the other hand, metabolize at a much slower rate, and have generally well-defined reserve proteins which, once formed, do not seem to undergo further metabolic changes until germination, so that one can assume that all the nitrogen which is taken in accumulates.

Previous work of Danielsson (1949*a*) in this laboratory on the pea proteins has shown that there are two well-defined globulins, namely, vicilin, with an S_{20} value of 8, and legumin, with S_{20} 12·5; and a less well-defined albumin fraction, showing two main components (*a* and *b*) with sedimentation coefficients in the neighbourhood of 1 and 4 respectively. The same author, using ultracentrifuging and electrophoresis as analytical tools, studied the breakdown of the high-molecular proteins during germination (Danielsson, 1951), and also the formation of these same proteins during ripening of the seeds (Danielsson, 1952).

Although the emphasis in the present work is placed upon the lower-molecular nitrogenous constituents of pea seeds, data on the formation of the high-molecular proteins seemed necessary to complete the picture of the overall nitrogen balance. Part of these latter data confirm and amplify results obtained in Danielsson's study, which was carried out at the same time as the present one but with a different variety of pea.

EXPERIMENTAL

Material. Two different varieties of seeds of *Pisum sativum* were used. For development of experimental methods, seeds of a green wrinkled garden pea at two different stages of development were taken. The peas designated as 'unripe' (U) were of the size generally used for canning; those designated as 'ripe' (R) were fully grown dried seeds.

For the analyses proper, a garden pea of the variety Fenomen was used. The peas were grown in season, under natural conditions. Because of the many irregularities in the Swedish climate, the different stages, designated I–VI, were selected on the basis of weight rather than age (Table 1). Owing to an unusually short summer the peas did not mature, and part of the original seed was used for analysis of ripe peas (stage VII). Samples were taken every 3 days, and after shelling the peas were sorted according to size, then ground in a Waring Blendor and lyophilized.

The dried material was finely ground and stored in glass bottles. It was sufficiently uniform to permit good reproducibility with Kjeldahl and Van Slyke analyses performed directly on the solids; there was no change when these analyses were repeated after several months' storage.

Analysis of the original seed material. Moisture determinations were made on the fresh peas and on the lyophilized material by drying at 75° for 3–4 days. If drying was continued for a longer time slight increases in weight were observed.

Total nitrogen was determined by micro-Kjeldahl on samples (10 mg.) of the lyophilized material; deviations ranged from 2 to 5%. For amino nitrogen determinations samples (50 mg.) were washed directly into the Van Slyke reaction chamber and reacted for 20 min.; consecutive determinations agreed within 1–3%.

Table 1. *Physical characterization of peas at different stages of development*

Stage	Wet wt. of 100 peas (g.)	Dry wt. of 100 peas (g.)
I	1·30	0·20
II	6·08	0·93
III	14·7	2·24
IV	27·8	5·40
V	45·8	10·17
VI	50·7	15·10
VII	22·2	20·99
U	42·5	11·30
R	27	24·60

Comparison of methods of extraction. Since it was important to obtain representative extracts, a large number of extractions were performed to determine the best conditions. The extractions were carried out by placing an accurately weighed amount (0·25–1·5 g.) of lyophilized material in a dry beaker, then adding a measured volume (25 ml.) of extractant and stirring for the required time. For extraction periods of 20 min., the solutions were kept at room temperature (22°); overnight extractions were performed at 4°. The resulting suspension was centrifuged at 3500 rev./min. for 10–15 min. and the supernatant filtered through a dry sintered-glass filter. When the solutions obtained in this manner were still turbid, they were rapidly frozen, kept overnight at −16°, then allowed to thaw and centrifuged again at 10 000 rev./min. for 5 min. By this method completely clear extracts were always obtained. A minimum of three samples of each solution was used for Kjeldahl determinations.

The buffers which were used have already been described by Danielsson (1949*a*); their compositions are as follows. 'Standard buffer', pH 6·8–7: 0·2 M-NaCl; 0·03 M-Na_2HPO_4; 0·02 M-NaH_2PO_4. 'Extraction buffer', pH 6·8–7: M-NaCl; 0·06 M-Na_2HPO_4; 0·04 M-NaH_2PO_4. 'Borate buffer', pH 8: 0·6 M-NaCl; 0·02 M-H_3BO_3; 0·005 M-$Na_2B_4O_7$. 'Standard buffer' was found to extract 81 ± 1 % of the total nitrogen in unripe peas and 71 ± 1 % of the total nitrogen in ripe peas. The extraction was practically complete in 20 min., since the additional amount of nitrogen extracted over a period of 16 hr. was insignificant (Table 5). An increase in the concentration of salt from 0·3 to 1·1 M ('extraction buffer') had no significant effect on the extraction yield, but sometimes tended to lower it; the same was true when borate buffer was used. When the NaCl in the 'standard buffer' was substituted by KCl or LiCl, the extraction yield could be increased to 93 % of the total nitrogen. The relative distribution of the different nitrogenous constituents in the extracts, however, remained the same (Table 5).

Dilute alkali was found to give the largest yield; for this reason such solutions have been extensively used in plant analyses, despite the fact that alkali is known to alter to some extent plant protein preparations (see, for example, Osborne, 1907; Blish & Sandstedt, 1929; Neglia, Hess & Sullivan, 1938). The use of alkali is unjustifiable, however, since it results in a complete breakdown of the proteins in the seed, as can be seen from the solid line in Fig. 1, which represents an ultracentrifuge diagram of a 0·1 % KOH extract of unripe peas, dialysed against distilled water, lyophilized and redissolved in standard buffer. With this information in mind, analytical results obtained with alkali extracts should be accepted with reservation. Even the painstaking and frequently quoted work of Emmerling (1880, 1887, 1900), who determined legumin and albumin in KOH extracts of peas, must be read in this light.

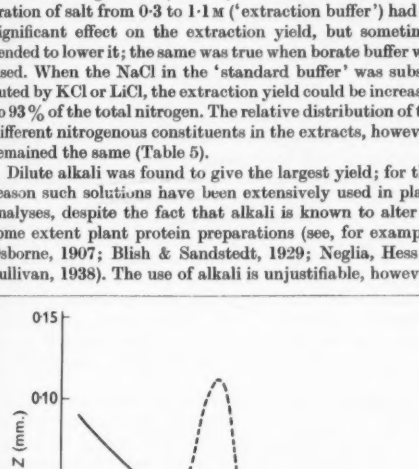


Fig. 1. Typical ultracentrifuge pattern of the proteins in extracts of the wrinkled green pea. – – –, 'Standard buffer'; ——, KOH. The ordinate is proportional to the gradient of refractive index; abscissa is distance from the centre of rotation.

Analysis of the extracts. The extracts, obtained by stirring the peas with 'standard buffer' for 20 min., were analysed for total nitrogen, protein nitrogen, total and 'protein' amino nitrogen, and total and 'protein' amide nitrogen. Total non-protein and peptide nitrogens were obtained by difference according to the following scheme:

Total N–Protein N = Total non-protein N,
Total amino N–'Protein' amino N = Amino N,
Total amide N–'Protein' amide N = Amide N.
Total non-protein N–(amino N + amide N) = Peptide N.

Four to six extracts of the peas in each developmental stage were analysed, except for stage I, for which only two extractions were made.

Total nitrogen (Kjeldahl) was determined on a minimum of three samples of each extract. The results were calculated by reference to the original volume of the extractant rather than to the volume of solution after extraction (Danielsson, 1951). This is not strictly accurate, but the error resulting from changes in volume upon the dissolution of the seed material is minimal. On the other hand, when the results are referred to the volume of solution measured after centrifuging or filtration of the seed material, the nitrogen in the solution retained by the precipitate (amounting to several millilitres) is excluded from analysis.

Since the quantitative determination of proteins, especially in the presence of polypeptides, presents great difficulties, several precipitation methods were checked against known quantities of different pea-protein fractions, whose ultracentrifuge patterns had been previously determined in order to verify their 'purity'. The globulins and albumins were prepared according to the method of Danielsson (1949*b*). Peptides U were prepared essentially according to the technique of Borsook, Deasy, Haagen-Smit, Keighly & Lowy (1949), after the extracts had previously been freed from legumin by isoelectric precipitation, and from vicilin and albumin by heat coagulation; this peptide fraction comprised the low-molecular albumin component (probably the proteose of earlier workers) and some smaller peptides. As can be seen from Table 2, no clear-cut separations could be obtained except with tungstic acid, which precipitated both proteins and peptides completely. The latter reagent was further tested with low-molecular peptides (Table 3) and on whole extracts; a comparison between these results and results obtained by dialysis is given in Table 4.

The incomplete precipitation of proteins by Stutzer's reagent confirms earlier observations (Hart & Bentley, 1915; Hiller & Van Slyke, 1922). This reagent is one most extensively used in plant analyses, and it should be noted that the results, although too low in terms of absolute values, might still be valid on a comparative basis but are in need of revision.

The precipitating efficiency of trichloroacetic acid (TCA) is dependent on the concentration of the original solution,

Table 2. *Comparison of various protein precipitants*

All solutions were allowed to stand for 2 hr. before centrifuging off the precipitate. U and R refer to preparations from unripe and ripe peas respectively.

Material	Concn. (mg. of N/ml.)	Reagent: 2·5% TCA	5% TCA	10% TCA	Cupric hydroxide†	Tungstic acid‡
		(N as % of total)				
Peptides U	0·113	68	—	—	—	100
Albumin R	0·157	80	88	95	—	100
Albumin U	0·105	78	76	82	—	100
Globulin	—	100	—	—	—	100
Peptides U	0·153	—	—	—	89	—
Albumin R	0·320	—	—	—	94	—
Albumin U	0·243	—	—	—	83	—
Peptides U*	0·133	23	—	73	—	—
Albumin R*	0·133	50	—	65	—	—

* The material was precipitated from 'standard buffer', instead of from distilled water.
† 1·5 ml. of 0·1 N-NaOH was added to 1 ml. of sample solution, followed by 1·5 ml. of 0·11 N-$CuSO_4$ (Blish, 1922).
‡ 1 ml. of solution was precipitated with 1 ml. of 10% sodium tungstate and 1 ml. of 0·1 N-H_2SO_4 (Hiller & Van Slyke, 1922).

Table 3. *Precipitation of asparagine and low-molecular peptides with tungstic acid*

Samples (2 ml.) of the extract were precipitated with 1 ml. of 10% sodium tungstate solution and 0·5 ml. of 6 N-H_2SO_4.

Material	Precipitation time	Concn. of N in soln. (mg. %)	N precipitated (%)
Asparagine	30 min.	17·8	37
	24 hr.	17·8	38
Glycyltyrosine	30 min.	9·75	15
	30 min.	9·75	15
	24 hr.	9·75	19
	24 hr.	9·75	17
Diglycylglycine	30 min.	19·8	27
	30 min.	19·8	27
	24 hr.	19·8	28
	24 hr.	19·8	28

Table 4. *Comparison of non-protein nitrogen values obtained by dialysis and by tungstic acid precipitation of extracts of whole peas*

Peas	Dialysis	Tungstic acid: 30 min.	16 hr.
		(N as % dry wt.)	
U	1·23	1·22	0·97
		1·26	0·96
		1·24	—
R	0·63	0·74	0·65
		0·72	0·62
Stage V	3·00	2·57	—
		2·42	—
Stage VI	2·60	1·90	—
		1·72	—

especially for the intermediate range of small proteins and large peptides (e.g. peptides U), and also on specific factors other than molecular size.

Tungstic acid produces complete precipitation of proteins and any intermediary products, but, according to Hiller & Van Slyke (1922), it does not precipitate amino acids; however, it cannot be used for separating either proteins and peptides from amino acids or proteins from amino acids and peptides, since it precipitates small peptides only partially (Table 3). When only small amounts of low-molecular diffusible peptides or asparagine or both are present, as they are in extracts of seeds U and R, the amount of non-protein nitrogen as determined by a rapid (30 min.) tungstic acid precipitation agrees well with the amount determined by dialysis (Table 4). If, however, a large quantity of peptides or asparagine or both is present (Table 8), the values for non-protein nitrogen as determined by tungstic acid will be low. Conversely, if protein is estimated by the determination of Kjeldahl nitrogen on the residues from tungstic acid extraction (Petrie & Wood, 1938), these values will be much too high because they incorporate not only all the unextracted nitrogen (10–30% of the total) but also a considerable percentage of the peptides present.

It is clear from the above that fractionations by precipitating agents depend upon many factors in addition to size. Moreover, since precipitates dissociate according to mass action, rigorously standardized conditions, including concentrations, are needed to yield meaningful results. Dialysis, on the other hand, offers a means of separation which depends more directly upon molecular size and is relatively less sensitive to changes in the compositions of the solutions used.

Accordingly, a simple procedure based upon dialysis was adopted in the present investigation. The extract (5 ml.) was placed in a thin cellophan bag (Visking casing) tied so as to have a compressed bubble of air above the liquid, and was dialysed against 300 ml. of 'standard buffer' (or other, depending upon the extract) for about 42 hr. No appreciable difference was noticed in the results if the extracts were dialysed for twice that time. After dialysis, the bags were opened and samples were taken for nitrogen determinations. Total Kjeldahl nitrogen of the dialysed solution was taken as protein nitrogen. The results were referred back to the original volume since the change in volume when dialysis is performed in a tightly closed bag, against the same buffer, is negligible.

Since non-protein nitrogen was taken as the difference between total nitrogen and protein nitrogen, a further

check on the procedure was made by determining nitrogen on samples of the diffusate as well as of the dialysed solution. The values for non-protein nitrogen obtained by the direct method were 0·69% for ripe peas and 1·18% for unripe ones, whereas those obtained by difference were 0·60% and 1·24% respectively. The values for protein nitrogen obtained by dialysis were independent of concentration, as can be seen from Table 5.

Amino nitrogen was determined by the method of Van Slyke, with a reaction time of 20 min. For determinations of amide nitrogen the samples were hydrolysed in approximately 6N-HCl (1 part of extract to 1 part of conc. HCl) for exactly 1 hr. at 95°. After hydrolysis, the sample was diluted, transferred to a Kjeldahl distillation flask, made alkaline with 30% NaOH, steam-distilled, and titrated in the usual manner. The method was tested with pure asparagine and compared with Kjeldahl nitrogen of the same solution. Total amino and amide nitrogen values were obtained from determinations on whole extracts, 'protein' amino and amide nitrogens from determinations on the dialysed solution. Two samples of each solution were analysed.

The values for 'protein' amino nitrogen show a certain inconsistency which suggests some abnormal reactivity toward the Van Slyke reagent, and therefore may well not represent true protein amino groups. The 'protein' amide nitrogen, on the other hand, is probably part of the protein, since it remains relatively constant in terms of percentage of total protein (6·5–8·4%).

Table 5. *Nitrogen distribution in extracts from ripe peas as a function of the extractant and concentration of the starting material*

Results are given in % of dry material.

Extractant	Starting material/100 ml. of extractant (g.)	Total N	Protein N	Non-protein N	Amino N
'Standard buffer' (20 min.)	6·17	3·30	2·82	0·48	0·34
	6·27	3·34	2·97	0·37	0·34
	6·30	3·32	2·57	0·75	—
	6·30	3·65	2·65	1·00	—
	6·05	3·58	2·88	0·70	—
	6·05	3·22	2·93	0·29	—
	6·05	3·22	2·94	0·28	—
	4·05	3·60	2·93	0·67	—
	4·15	3·45	2·87	0·58	—
	2·02	3·21	2·67	0·54	—
	2·05	3·32	2·86	0·46	—
	2·01	3·27	2·63	0·64	—
	1·00	3·30	2·93	0·37	—
	1·02	3·35	3·01	0·34	—
	Average	3·36	2·83	0·53	0·34
'Standard buffer' (16 hr.)	6·40	3·68	2·83	0·85	—
	6·22	3·46	2·85	0·61	—
	1·09	3·62	3·21	0·41	—
	Average	3·59	2·96	0·63	0·34
0·1% KOH	2·11	3·97	3·45	0·51	—
	1·03	3·82	3·46	0·47	—
	Average	3·90	3·45	0·49	—

Table 6. *Distribution of protein in extracts*

Component ...	Legumin		Vicilin		Albumins	
	R	U	R	U	R	U
			(% of total protein)			
	9	19	59	64	32	17
	10	15	55	68	35	17
	23	17	43	61	34	27
	20	15	47	49	33	36
	21	24	52	52	27	24
	23	24	50	53	27	23
	26	24	48	54	26	22
	24	28	41	56	35	16
	25	17	48	59	27	24
Average	20±1·6*	21±1·4	49±1·3	57±1·6	31±1·0	23±1·4

* Standard error.

The determination of peptide by difference is open to objection on the ground that it includes any undetermined soluble nitrogenous compounds which might be present. The concentration of these products, however, is so small that no serious error can be introduced. The spread of results in this fraction would be expected to be large, since it reflects the accumulation of errors made in the other determinations. The average of a number of determinations, however, is useful to indicate the trend which this fraction follows.

The presence of peptides has been demonstrated more directly in several ways. In one experiment, the diffusate of a 'standard buffer' extract was concentrated and treated with 10% TCA. A small amount of precipitate was obtained, which gave one immobile and several mobile ninhydrin-positive spots on a paper chromatogram, with propanol–butanol–0·1 N-HCl (2:1:1) as the solvent. In another experiment an aqueous extract was electrodialysed, the precipitated protein removed by centrifuging and the three compartments were chromatographed separately. In all three, a large number of spots unaccountable for by amino acids were obtained.

The concentration of the different protein components in the extracts were determined by measuring the corresponding areas in the ultracentrifuge diagrams, according to Danielsson (1951). This method is not very exact, as can be seen from the data given in Table 6, and requires a large number of determinations for accurate results; since such were not critical for the purposes of the present investigation, ultracentrifuging was carried out four or five times for each stage of development.

RESULTS

The distribution of total and amino nitrogen in whole peas is given in Table 7. The nitrogen distribution in the extracts is given in Table 8. Fig. 2 represents a comparison between extracted and unextracted total and amino nitrogens respectively. The curves are sufficiently parallel to justify accepting the results obtained with extracts as representative of the whole seeds.

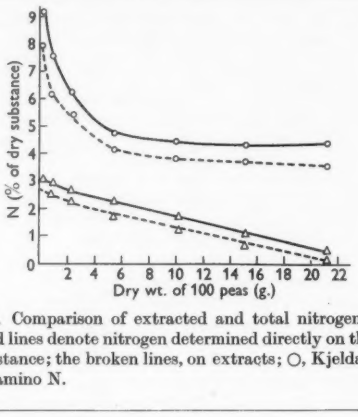


Fig. 2. Comparison of extracted and total nitrogen. The solid lines denote nitrogen determined directly on the dry substance; the broken lines, on extracts; ○, Kjeldahl N; △, amino N.

Table 7. *Distribution of total and of amino nitrogen in fresh and dry peas at different stages of maturity*

Stage	N as % wet wt.		N as % dry wt.		N as mg./100 peas		Moisture (%)
	Total	Amino	Total	Amino	Total	Amino	
I	1·41	0·46	9·24	3·03	18·5	6·1	84·7
II	1·16	0·44	7·60	2·85	70·7	26·5	84·7
III	0·96	0·39	6·29	2·54	140·9	56·9	84·7
IV	0·92	0·42	4·74	2·14	256·0	115·6	80·6
V	0·99	0·38	4·45	1·73	452·6	175·9	77·8
VI	1·30	0·32	4·38	1·06	661·4	160·1	70·2
VII	4·16	0·39	4·46	0·42	936·2	96·6	6·7
U	1·35	0·14	4·93	0·54	550	61	73·8
R	4·32	0·36	4·72	0·39	1160	97	8·5

Table 8. *Nitrogen distribution in extracts of peas in different stages of maturity*

Percentages were corrected for moisture content of the lyophilized material.

Stage	Total N	Protein N	Non-protein N			
			Total	Amino	Amide	Peptide*
	(% dry wt.)					
I	8·17	1·54	6·63	2·65	0·6	3·4
II	6·25	1·48	4·87	2·52	0·7	1·6
III	5·39	1·02	4·37	2·21	0·7	1·4
IV	4·17	0·77	3·40	1·75	0·5	1·2
V	3·93	0·79	3·14	1·27	0·5	1·4
VI	3·79	1·36	2·43	0·71	0·2	1·6
VII	3·56	2·95	0·61	0·12	0·1	0·4

* Obtained by difference.

In order to assure a clear picture of the development of the seeds, results are given, not only as percentage of dry substance, but also as percentage of original (wet) material, and as mg. of nitrogen/100 seeds. It is important to know the absolute amounts of nitrogenous materials during the development of the individual seed, in order to bring out the manner in which the incoming nitrogen is used for synthesis, and to follow possible interconversions of nitrogenous fractions.

It can be seen from Table 7 that, whereas the concentration of amino nitrogen in the fresh seed (calculated as percentage of wet weight) remains quite constant throughout the entire period of development, the concentration of total nitrogen decreases at first and later increases markedly. This rapid increase begins at stage V and corresponds to the onset of desiccation, as may be seen from the last column of Table 7. From Table 8 it can be seen that on a dry-weight basis the concentrations of all components, with the exception of protein, decrease as the seed ripens. More eloquent, however, is the absolute distribution of the different nitrogen fractions, as represented by Fig. 3. It can be seen that there is a uniform influx of nitrogen into the seed which, at first, is distributed more or less uniformly among the fractions. At stage V amino and amide nitrogen begin to decrease, while peptide and protein nitrogen continue to increase. At stage VI, peptide nitrogen also begins to decline, and only protein continues the sharp increase. At the same time there is a sharp increase in the 'protein' amino and amide nitrogen, as shown in Table 9.

The distribution of the protein components in the extract is given in Table 10. The figures represent averages of at least four runs on different extracts. Fig. 4 shows characteristic ultracentrifuge diagrams of extracts, stages IV–VII. Because of the preponderance of albumin in the earlier stages, no satisfactory diagrams could be obtained; convection could not be eliminated and, further,

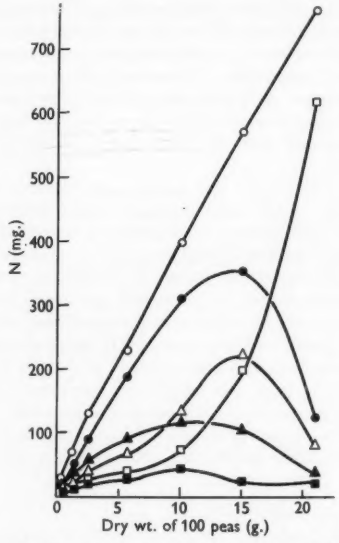


Fig. 3. Absolute nitrogen distribution in extracts from peas throughout the maturation process. The experimental points denote the different developmental stages (I–VII) used for the analyses: ○, total N; □, protein N; ●, total non-protein N; ▲, amino N; △, peptide N; ■, amide N.

Table 9. *Distribution of protein and free amino and amide nitrogen in pea extracts*

	Per cent of total amino N		Per cent of total amide N	
Stage	'Protein' amino N	Free amino N	'Protein' amide N	Free amide N
I	0	100	14	86
II	0	99	11	89
III	5	95	10	90
IV	6	94	10	90
V	8	92	11	89
VI	11	89	43	57
VII	54	46	83	17

Table 10. *Distribution of legumin, vicilin and albumin in extracts of peas in different stages of development*

	Total protein % of dry substance	Per cent of total protein		
Stage		Legumin	Vicilin	Albumin
III	6·4	—	—	100
IV	4·8	(7)	11	83
V	4·9	9	29	60
VI	8·5	16	46	38
VII	18·4	37	46	17

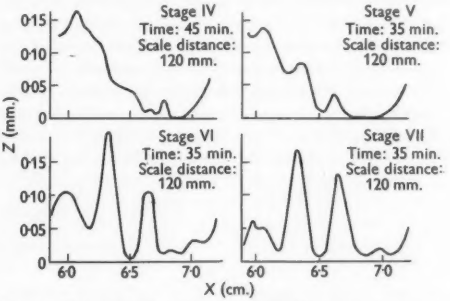


Fig. 4. Characteristic ultracentrifuge diagrams of extracts from peas at different stages of development. Coordinates as in Fig. 1.

sporadic peaks, probably due to association products, were observed. In stage III the bulk of the material is found in the albumin peak (which is not well differentiated), and neither vicilin nor legumin can be detected. There are, however, high-molecular components (S_{20} 18–20) present, which are believed to be association products of legumin (Danielsson, 1951).

In stage IV the vicilin peak is discernible for the first time. The peak ahead of vicilin is probably not legumin, although its appearance is persistent. Its sedimentation coefficient is in the neighbourhood of 16, but this in itself is not of great significance; owing to the small concentration of these components, the sedimentation coefficients are unreliable. More significant evidence against the peak being due to legumin is the low ratio of vicilin to legumin at this stage, as compared with subsequent ones. In stage V, vicilin and legumin are clearly present. It is difficult to determine the concentration, however, since the vicilin is poorly differentiated from the large albumin peak.

Analyses of peas U and R indicate that there is an increase rather than a decrease of albumin in the last stage of ripening (Table 6); furthermore, this increase was localized entirely in the lower molecular component (S_{20} approx. 1) of the albumin fraction and was coupled with a corresponding decrease in the peptide fraction (Table 11). The apparent discrepancy between this observation and the results obtained in the second series of analyses is easily explained by the fact that peas U were much closer to maturity than peas of stage VI, as can be seen from the values for protein and amino nitrogen.

DISCUSSION

A survey of the foregoing data permits the drawing in some detail of a picture of the fate of the nitrogenous compounds entering the pea seed throughout the process of its growth and ripening. One striking feature, which is brought out in Table 8, is that after the initial stages the amount of nitrogen entering the seed remains proportional to the amount of total dry matter during its whole development. However, at a certain stage, corresponding to the onset of water loss, the seed develops a tendency towards increased condensation, as reflected in the falling off of the values for the fractions representing non-protein nitrogen.

The changes in the three main nitrogenous fractions, amino nitrogen, peptide nitrogen and protein nitrogen (Fig. 3), are clearly suggestive of curves representing consecutive reactions. The phase of high 'condensing capacity' is marked at the beginning by a progressive increase in the peptide nitrogen at the expense of the amino nitrogen, and later by a striking increase in the protein nitrogen at the expense of the peptide nitrogen.

While it might clearly be misleading to press too far any analogy between a simple kinetic system and a complex plant organ, it is tempting to suggest that so far as these data bear on the problem of the mechanism of protein synthesis in pea seeds, the simplest interpretation would be in terms of a stepwise build-up of the proteins. It might be conceivable that the peptides are broken down to amino acids before utilization in the synthesis of the proteins; this is somewhat unlikely, however, since it would be necessary to assume the greatest degree of hydrolysis of peptides precisely at a time when the 'condensing capacity' of the seed is reaching its highest level, and when rapid loss of water (from 70·2 to 6·7 % moisture) would not seem to favour hydrolysis.

Curves typical of consecutive reactions, and very similar in character to those of Fig. 3 were obtained by Turba & Esser (1955) for the incorporation of ^{14}C into the different nitrogenous fractions of actively growing yeast cells. These authors isolated about 40 peptides, and in short-term experiments were able to demonstrate that the radioactive label was incorporated into peptides before it appeared in proteins.

Acceptance of the hypothesis of a stepwise build-up of the seed proteins would lead one to expect well-defined intermediates to fill the gap between the simple peptides and the globulins, but the question of the nature of these intermediates cannot at present be answered, although there are several points of evidence in favour of the formation of the globulins via albumin. The albumin fraction is extraordinarily heterogeneous and could thus be thought of as a pool of protein fragments varying greatly in size and amino acid composition.

In the beginning of seed formation, all the protein is in the form of albumin, the globulins appearing

Table 11. *Nitrogen distribution in pea extracts*

		Protein N (% dry wt.)					Non-protein N (% dry wt.)		
					Albumin				
Peas	Total N	Total	Legumin	Vicilin	*a*	*b*	Total	Amino	Peptide
U	3·96	2·72	0·54	1·55	0·29	0·34	1·24	0·46	0·78
R	3·36	2·83	0·57	1·39	0·55	0·32	0·53	0·34	0·19

only at stage IV. This would seem to indicate that a certain concentration of low-molecular protein has to be built up before synthesis of globulin can proceed. Once this level has been reached, globulins can be formed even in detached unripe seeds, which at first show only the albumin peak. As has been shown by Danielsson (1952) this appearance of globulin is associated with little or no change in amino nitrogen. This suggests that the combining fragments are of rather high molecular weight, which in turn means that they must appear in the ultracentrifuge diagrams located in the broad 'albumin' peak. The second point of evidence is that the final increase in protein just before desiccation is not distributed uniformly over all protein fractions, but is concentrated in the low-molecular albumin peak (Fig. 5, Table 11). Unfortunately, time has not yet permitted us to confirm this very interesting finding by analysing the albumin fractions of a different series of seeds, but the observation is, nevertheless, highly suggestive. It should be pointed out that the rapid increase of globulins as compared with a very gradual increase of albumins (Danielsson, 1952) does not argue against the latter being intermediates. On the contrary, since the globulins are largely insoluble in the seed, one would expect the equilibrium to be in their favour, the albumins being left at a more or less constant level.

Since the arguments against a stepwise synthesis of protein rely so heavily on the inability of many workers to demonstrate peptide intermediates or even to detect peptides in tissues in general, it might be appropriate to comment on this difficulty here.

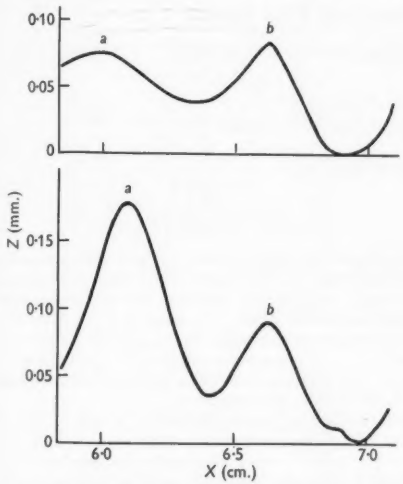


Fig. 5. Ultracentrifuge diagrams of the albumin fraction of peas, Upper, unripe peas (U); lower, ripe peas (R). Co-ordinates as in Fig. 1.

One reason that peptides have not been found more frequently is undoubtedly that the deproteinization procedures commonly used remove the peptides together with the proteins. It has been seen, for example (Table 3), that tungstic acid precipitates even small peptides to a considerable extent. Moreover, as has been pointed out by Turba & Esser (1955) peptides have a great tendency to be adsorbed on to proteins and are thus precipitated with the latter even by reagents such as trichloroacetic acid, which normally would leave the peptides in the supernatant fluid. Many peptides also behave in an unconventional manner in extraction procedures. Deane & Truter (1955), for example, report that there are many peptides in suint that are soluble in 70–80 % alcohol, and therefore would be discarded with the amino acids fraction. Furthermore, these peptides, containing from 8 to 13 amino acids, cannot be distinguished from known amino acids in several two-dimensional chromatographic systems, and could only be revealed as peptides after hydrolysis and re-chromatography.

From the few examples given it appears that the problem of the natural occurrence of peptides in biological systems needs to be re-examined. The method described in the present paper might give valuable leads in the search for these compounds, although the quantitative data might be somewhat in error because of the indirect nature of the determinations. It should be remembered that if labile amides are present which give off the same nitrogen atom in both the amide and the Van Slyke analyses, the values for peptide nitrogen, obtained by difference, would be low. This error then would compensate some of the error in the opposite direction due to nitrogen of bases other than amino acids and to the non-amino nitrogen of the basic amino acids. The apparently high concentration of peptide nitrogen in the very young seeds (stage I) might, in part, be due to the high arginine content of such seeds (Schulze & Winterstein, 1910; E. W. Yemm, private communication); but since glutamine is also present in considerable amounts, the error, at least in part, would be corrected. Similar errors, however, cannot alter significantly the values in stages VI and VII, where the peptide nitrogen exceeds other soluble nitrogen (Table 8). Moreover, it is not so much individual values, as it is the whole trend of the peptide fraction (and especially the increase in peptide concentration in the peas of stages V and VI, and the subsequent decrease in stage VII) that is important for the conclusions drawn in the present paper, and that also would be important for the investigation in other systems of the role of peptides in protein synthesis.

SUMMARY

1. Extracts of *Pisum sativum* at seven different stages of development were analysed for total nitrogen, non-dialysable nitrogen, total and non-dialysable amino nitrogen and total and non-dialysable amide nitrogen; total non-protein nitrogen and peptide nitrogen were determined by difference.

2. The protein distribution in the extract was determined from ultracentrifuge diagrams.

3. In the early stages of development the incoming nitrogen was distributed about equally among the different fractions. With ensuing desiccation, however, there occurred first a sharp increase in peptide nitrogen and later a sharp increase in protein nitrogen at the expense of peptide nitrogen.

4. The data offer strong evidence in favour of peptide intermediates in the synthesis of the seed protein.

REFERENCES

Bisson, C. S. & Jones, H. A. (1932). *Plant Physiol.* **7**, 91.

Blish, M. J. (1922). *J. biol. Chem.* **53**, 251.

Blish, M. J. & Sandstedt, R. M. (1929). *J. biol. Chem.* **85**, 195.

Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighly, G. & Lowy, P. H. (1949). *J. biol. Chem.* **179**, 705.

Boswell, V. R. (1924). *Proc. Amer. Soc. hort. Sci.* **21**, 178.

Chibnall, A. C. (1939). *Protein Metabolism in the Plant.* Yale: University Press.

Danielsson, C. E. (1949*a*). *Biochem. J.* **44**, 387.

Danielsson, C. E. (1949*b*). *Acta chem. scand.* **3**, 41.

Danielsson, C. E. (1951). *Acta chem. scand.* **5**, 541.

Danielsson, C. E. (1952). *Acta chem. scand.* **6**, 149.

Deane, K. R. & Truter, E. V. (1955). *Biochim. biophys. Acta*, **18**, 435.

Emmerling, A. (1880). *Landw. Versuchsw.* **24**, 113.

Emmerling, A. (1887). *Landw. Versuchsw.* **34**, 1.

Emmerling, A. (1900). *Landw. Versuchsw.* **54**, 215.

Hart, E. B. & Bentley, W. H. (1915). *J. biol. Chem.* **22**, 477.

Hiller, A. & Van Slyke, D. D. (1922). *J. biol. Chem.* **53**, 251.

Nedokutschajew, N. (1902). *Landw. Versuchsw.* **56**, 303.

Nedokutschajew, N. (1904). *Landw. Versuchsw.* **58**, 275.

Neglia, F. J., Hess, W. C. & Sullivan, M. X. (1938). *J. biol. Chem.* **125**, 183.

Osborne, T. B. (1907). *Publ. Carneg. Instn*, no. 84.

Petrie A. H. K. & Wood, J. G. (1938). *Ann. Bot., N.S.*, **2**, 33.

Schulze, E. (1911). *Hoppe-Seyl. Z.* **71**, 31.

Schulze, E. & Winterstein, E. (1910). *Hoppe-Seyl. Z.* **65**, 431.

Steward, F. C. & Preston, G. (1940). *Plant Physiol.* **15**, 23.

Steward, F. C. & Preston, G. (1941). *Plant Physiol.* **16**, 85.

Turba, F. & Esser, H. (1955). *Biochem. Z.* **327**, 93.

Wood, J. G. & Petrie, A. H. K. (1942). *Aust. J. exp. Biol. med. Sci.* **20**, 249.

Zaleski, W. (1905). *Ber. dtsch. bot. Ges.* **23**, 126.

Protein Synthesis in Ripening Pea Seeds

2. DEVELOPMENT OF EMBRYOS AND SEED COATS

By ILSE DOROTHEA RAACKE*

Biochemical Institute, University of Uppsala, Sweden

(*Received* 22 *June* 1956)

In seeds of the Leguminosae, the constituent parts do not all develop simultaneously. For example, it is known that the endosperm develops first, only to be later absorbed by the developing embryo (including the cotyledons). This means that while protein is being built up in some parts of the seed, it is being broken down in others, so that a study of the development of the whole seed gives only the net result of protein formation. A truer picture might be obtained by studying independently the development of different parts of the seed. As far as the author is aware, such a study has not previously been carried out.

* Present address: Chemical Laboratory, University of Cambridge.

EXPERIMENTAL

The material consisted of peas of the same lot as those used in the preceding paper (Raacke, 1957). They were grouped into stages as previously described, but the groups represented here are not exactly the same as those of Part 1 of this investigation, although they roughly correspond. The embryos of peas belonging to stages I, II and III were too small to be separated, so that only embryos of peas of stages IV, V, VI and VII were used.

The seed coats of peas in stages IV, V and VI were removed by making a small incision in the coat, through which the embryo could be squeezed out. The ripe peas, stage VII, were allowed to soak overnight in water at 2° before removal of the coat.

The material designated as 'cotyledon IV' is a whole embryo of peas belonging to stage IV. From peas at stages V,

VI and VII, the hypocotyls and epicotyls were dissected out and only the cotyledons used. The hypocotyls of stages V and VI were pooled and designated as 'Hyp'.

The seed coats, cotyledons and hypocotyls were minced in a Waring Blendor and lyophilized in the manner described in Part 1 of this investigation. The lyophilized material was then analysed for moisture, Kjeldahl and amino nitrogen, and extracted with 'standard buffer'. The extracts were analysed as previously described (Raacke, 1957).

RESULTS

A comparison between Kjeldahl and amino nitrogen of cotyledons and Kjeldahl and amino nitrogen of seed coats is given in Table 1. It is seen that both become relatively poorer in nitrogen as ripening progresses.

Table 2 gives the nitrogen distribution in extracts of the different seed parts. As in the previous paper, amino and amide nitrogen represent dialysable forms only, and amino and peptide nitrogen represent nitrogen in amino and in peptide links respectively. The figures show a sharp increase of protein and a decrease of amino, amide and peptide nitrogen in the cotyledons as maturity is approached. In the beginning (stage IV) only 29 % of the total nitrogen is present as protein, 31 % as amino, 31 % as peptide and 9 % as amide nitrogen. In the ripe cotyledons, however, 82 % of the total nitrogen is present as protein, and only 5 % as amino, 13 % as peptide and none as amide nitrogen. Fig. 1 shows the absolute distribution of nitrogen in the extracts of cotyledons. It is seen that, as in the picture for whole peas, there is a divergence of the curves representing protein and non-protein nitrogens between stages V and VI.

In the seed coats, in contrast to the cotyledons, the relative distribution of the different nitrogenous fractions remains relatively constant throughout.

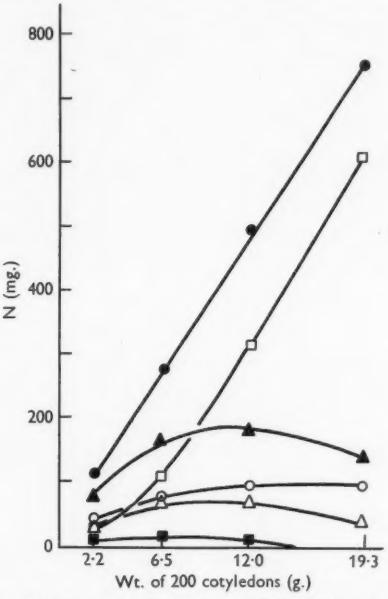


Fig. 1. Nitrogen distribution in extracts of cotyledons at different stages of ripening. ●, Total N; □, protein N; ▲, total non-protein N; ○, peptide N; △, amino N; ■, amide N.

Table 1. *Partial nitrogen analysis of cotyledons and seed coats*

Peas (stage)	Cotyledons		Seed coats		Hypocotyls	
	Total N	Amino N	Total N	Amino N	Total N	Amino N
			(N as % dry wt.)			
IV	5·81	2·23	3·56	1·45	7·69	1·00
V	4·99	1·46	3·10	0·88	—	—
VI	4·87	0·83	2·26	0·64	—	—
VII	4·45	0·35	0·66	—	—	—

Table 2. *Nitrogen distribution in extracts of cotyledons and of seed coats*

Material	Stage	Total N	Protein N	Non-protein N			
				Total	Amino	Amide	Peptide
				(% dry wt.)			
Cotyledons	IV	5·11	1·48	3·63	1·57	0·49	1·57
	V	4·25	1·69	2·56	1·09	0·21	1·26
	VI	4·14	2·65	1·49	0·60	0·08	0·81
	VII	3·90	3·18	0·72	0·21	0	0·51
Seed coats	IV	2·94	0·43	2·51	1·38	0·11	0·89
	V	2·27	0·39	1·88	0·86	0·09	0·93
	VI	1·70	0·24	1·46	0·62	0·09	0·75
	VII	0·18	0·07	0·11	0·03	0·02	0·06
Hypocotyls	—	—	1·33	1·51	0·56	—	—

About 15 % of the nitrogen is present as protein, 40 % as amino, 40 % as peptide and 5 % as amide nitrogen. There seems to be a depletion of the soluble nitrogen forms in the ripe seed coats, but the figures are too low to be trustworthy.

The distribution of free and bound amino nitrogen, and free and bound amide nitrogen in the cotyledons and seed coats respectively is shown in Table 3. The bound amino nitrogen in the cotyledons is found to increase from about 7 % of the total amino nitrogen in the unripe seeds to 40 % of the total in ripe ones. During the same period the bound amide nitrogen increases from 20 to 100 % of the total amide nitrogen. In the seed coats, the amino nitrogen distribution remains approximately constant, but there is a constant decrease in the bound amide nitrogen.

The protein distribution in the extracts of cotyledons was determined from ultracentrifuge diagrams. The results are given in Table 4 and Fig. 2 shows typical diagrams of the four stages investigated. The seed coats contain only albumin; the different stages were not investigated separately.

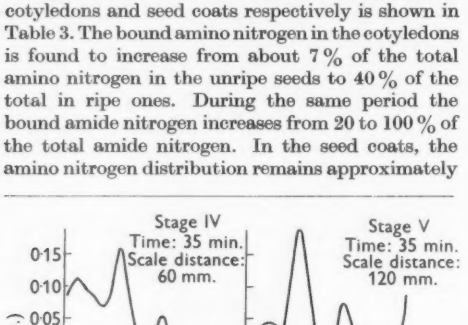


Fig. 2. Representative ultracentrifuge diagrams of extracts of cotyledons at four different stages of development. The ordinate is proportional to the gradient of refractive index; abscissa is distance from the centre of rotation.

DISCUSSION

Separate analyses of the development of the seed coats and of the cotyledons show that the maturation process as observed with whole peas represents the net result of two superimposed trends. In the seed coats, although synthesis is undoubtedly occurring, the breakdown of protein is predominant. The figures in Table 2 show very clearly how the nitrogenous materials from the seed coats (comprising also the endosperm) are gradually absorbed by the developing embryo, thus corroborating chemically the long-known histological observations. In the cotyledons, on the other hand, the balance of metabolic activities lies overwhelmingly on the synthetic side, as is shown by the rapid increase in protein (Fig. 1). The marked rise and subsequent drop in the concentration of peptides observed previously with whole peas (Raacke, 1957), is due largely to the peptide fraction in the seed coats, the peptide level in the cotyledons remaining relatively constant. It would appear, then, that an important function of the seed coats is in the maintenance of a definite peptide pool in the cotyledons. Failure to maintain this pool at an appropriate level, as occurs in the last stage of

Table 3. *Free and bound amino and amide nitrogen in extracts of cotyledons and of seed coats*

		Amino N (% dry wt.)			Amide N (% dry wt.)		
Material	Stage	Total	Bound	Free*	Total	Bound	Free*
Cotyledons	IV	1·68	0·11	1·57	0·61	0·12	0·49
	V	1·23	0·14	1·09	0·35	0·14	0·21
	VI	0·72	0·12	0·60	0·28	0·20	0·08
	VII	0·33	0·14	0·21	0·30	0·30	0
Seed coats	IV	1·45	0·17	1·38	0·24	0·13	0·11
	V	0·92	0·06	0·86	0·11	0·02	0·09
	VI	0·67	0·05	0·62	0·12	0·03	0·09
	VII	0·03	0	0·03	0·02	0	0·02

* Difference between amino N before and after dialysis of the extracts.

Table 4. *Protein distribution in extracts of cotyledons*

Stage	Legumin	Vicilin	Ratio: vicilin/legumin	Albumin	Ratio: albumin/globulin
	(% of total protein)			(% of total protein)	
IV	15	44·5	2·94	40·5	0·68
V	25·5	54	2·12	20·5	0·26
VI	37	59	1·59	4	0·04
VII	36	46	1·28	16	0·19

maturation when the seed coats are depleted of nitrogenous materials, causes protein synthesis to stop.

The picture of a constant-size peptide pool fits in well with the hypothesis of definite intermediate stages in the build-up of high-molecular proteins, since on one side there is the well-established necessity for an adequate amino acid pool within the cell, and on the other, a build-up and maintenance of a certain size albumin pool as a preliminary for the synthesis of the pea globulins.

The development of the different protein components can be seen more clearly in the cotyledons than in the whole peas, because in this case the seed coat with its large amount of albumin is eliminated. It is again found that vicilin appears first. From the diagrams there is also clearly discernible a decrease of the concentration of albumin and then again a final increase. This confirms an observation reported in the previous paper. This final increase is not evident from the series of analyses on whole peas since, as was mentioned above, the albumin from the seed coats obscures the picture. It should be noted that Danielsson (1952) also observed an increase of the albumin/globulin ratio in the last stage.

The figures in Table 3 are notable as they show the gradual and ultimately complete incorporation of the amide nitrogen into the protein of the cotyledons. It has often been suggested that amide occupies a pivotal position in the nitrogen metabolism of the seed (Schulze, 1900; Schulze & Castoro, 1903; Prianischnikow, 1922; Mothes, 1926; Vickery, Pucher & Clark, 1936; Vickery & Pucher, 1943; Gregory & Sen, 1937; Steward & Preston, 1941). From the present data, however, it appears that amide is also utilized directly and in much the same manner as other amino acids in the build-up of the pea proteins. This, of course, does not mean that amides do not have any special functions, since there is, indeed, much evidence that these compounds serve as a storage product of ammonia. But it serves to emphasize that amide metabolism in the plant does not follow a single pathway, and that in normally synthesizing tissues, which are not subjected to any stress such as etiolation or unbalanced supply of nutrients which calls upon the defence mechanism of the plant, at least part of the amide nitrogen does not suffer a fate different from that of the amino nitrogen.

SUMMARY

1. Cotyledons and seed coats of *Pisum sativum* at four different stages of development have been analysed. It is shown that as ripening progresses the nitrogenous materials from the seed coats (including the endosperm) are absorbed by the cotyledons.

2. The study of the nitrogen distribution in the cotyledons corroborates the evidence obtained from studies of whole peas to the effect that protein synthesis proceeds through various stages which include peptides and 'proteoses'.

3. It was found that in cotyledons amide nitrogen is incorporated gradually but completely into the protein; the relationship of this finding to current views of amide metabolism in plants is discussed.

REFERENCES

Danielsson, C. E. (1952). *Acta chem. scand.* **6**, 149.
Gregory, F. G. & Sen, P. K. (1937). *Ann. Bot., N.S.*, **1**, 521.
Mothes, K. (1926). *Planta*, **1**, 472.
Prianischnikow, D. (1922). *Ber. dtsch. bot. Ges.* **40**, 242.
Raacke, I. D. (1957). *Biochem. J.* **66**, 101.
Schulze, E. (1900). *Hoppe-Seyl. Z.* **30**, 241.
Schulze, E. & Castoro, N. (1903). *Hoppe-Seyl. Z.* **38**, 199.
Steward, F. C. & Preston, C. (1941). *Plant Physiol.* **16**, 85.
Vickery, H. B. & Pucher, G. W. (1943). *J. biol. Chem.* **150**, 197.
Vickery, H. B., Pucher, G. W. & Clark, H. E. (1936). *Plant Physiol.* **11**, 413.

Protein Synthesis in Ripening Pea Seeds

3. STUDY OF THE PODS

By ILSE DOROTHEA RAACKE*
Biochemical Institute, University of Uppsala, Sweden

(*Received* 22 *June* 1956)

It is well known that there is a translocation of nutrients from the pods of the Leguminosae to the developing seeds. This was first observed by Emmerling (1880) for *Pisum sativum* and later confirmed by Schulze & Winterstein (1910) and Bisson & Jones (1932), as well as by Wassilieff (1908), for *Lupinus albus*, and by Pfenninger (1909) for *Phaseolus vulgaris*. The available evidence indicates that the nitrogenous nutrients do not pass directly from the leaves to the seeds through the pods, but are at least in part at first stored in the pods in the form of protein, which is then used to supply the developing seeds.

* Present address: Chemical Laboratory, University of Cambridge.

Many workers have studied the transfer of the pod proteins to the seeds, but although it is certain that the proteins must be broken down before translocation, the form in which the nitrogen passes to the seeds is still rather uncertain. Because of large amounts of asparagine present in the stems and pods of several species of Leguminosae, Schulze & Winterstein (1910) concluded that nitrogen was translocated in the form of that amide, rather than in the form of amino acids. However, in addition to these two possibilities, it is also conceivable that peptides play a part in the transfer; hence, it seemed of interest to determine peptide nitrogen in the developing pods.

EXPERIMENTAL AND RESULTS

The experimental material for the present investigation consisted of pods of *Pisum sativum*, variety Fenomen, grown and sampled as described in Part 1 of this investigation (Raacke, 1957). The pods were divided into seven groups according to the physical characteristics given in Table 1. Unfortunately, it was not possible to obtain ripe, i.e. dry pods, so that all seven groups represent immature stages.

The stages do not correspond to the stages of peas described previously (Raacke, 1957), because the peas were regrouped after shelling. So, for example, pods I yielded only peas I, but pods II yielded peas I and some II, pods III gave peas I and II and some III, and so on; pods VII yielded mainly peas VI, and some V and IV.

The pods were minced and lyophilized as previously described. In the more mature pods, long fibres which could not be minced were sifted out and discarded. The lyophilized material was subjected to moisture, Kjeldahl, and Van Slyke determinations, the results of which are given in Table 2.

Extractions were carried out with standard buffer and the extracts analysed as previously described. The extraction yields varied from 74 to 84 % of the total nitrogen and 76–88 % of the amino nitrogen of the dry material, so that the results obtained from the extracts can be taken as representative. The relative nitrogen distributions can be seen in Table 3. Fig. 1 shows the changes in the absolute amounts of the various nitrogenous fractions in the pods as maturation proceeds.

Ultracentrifuging of the extracts was also carried out. Because of the low protein content of the pods, the extracts were dialysed, lyophilized and the protein redissolved in 'standard buffer'. There was no obvious difference between the different stages,

Table 1. *Physical characteristics of pods of peas at different stages of development*

Stage	Wet wt. (g.)	Dry wt. (g.)	Length of pods (cm.)	Thickness of pods (cm.)
I	23	3·6	2–3	0·1
II	48	5·1	3·5–4·5	0·3
III	106	10·2	5·0–5·5	0·3–0·4
IV	285	29·4	6·0–7·5	0·4
V	336	36·0	8·0–9·0	0·4
VI	338	36·0	7–9	0·6
VII	337	36·0	7–8	0·8–0·9

Table 2. *Partial analysis of pods of* Pisum sativum *during maturation*

Pods (stage)	Total N		Amino N		Total N/100 pods (mg.)	Amino N/100 pods (mg.)	Moisture (%)
	Wet (%)	Dry (%)	Wet (%)	Dry (%)			
I	0·94	7·09	0·21	1·62	255	58	86·7
II	0·57	5·34	0·20	1·85	272	94	89·4
III	0·47	4·87	0·16	1·70	497	173	90·4
IV	0·42	4·60	0·16	1·83	1352	538	89·6
V	0·40	4·57	0·16	1·87	1645	673	89·3
VI	0·41	4·56	0·15	1·69	1642	609	89·5
VII	0·35	4·11	0·12	1·36	1480	490	89·5

Table 3. *Nitrogen distribution in extracts of pods during their development*

Stage	Total N	Protein N	Non-protein N			
			Total	Amino	Amide	Peptide
	(% dry wt.)					
I	4·88	1·65	3·23	1·20	1·05	0·98
II	4·26	1·02	3·24	1·56	0·93	0·75
III	3·71	0·79	2·92	1·34	0·91	0·67
IV	3·56	0·68	2·88	1·30	0·88	0·70
V	3·83	0·83	3·00	1·53	0·83	0·64
VI	3·45	0·53	2·92	1·28	0·83	0·77
VII	3·41	0·59	2·82	1·09	0·85	0·88

the bulk of the protein being contained in an ill-defined albumin peak with low sedimentation coefficient ($\simeq 1$–2), although a small peak of high sedimentation coefficient (16–22) was consistently present. This may be present originally in the pod, but more probably it is an aggregation product, as the varying sedimentation coefficients would tend to indicate. A typical diagram is shown in Fig. 2.

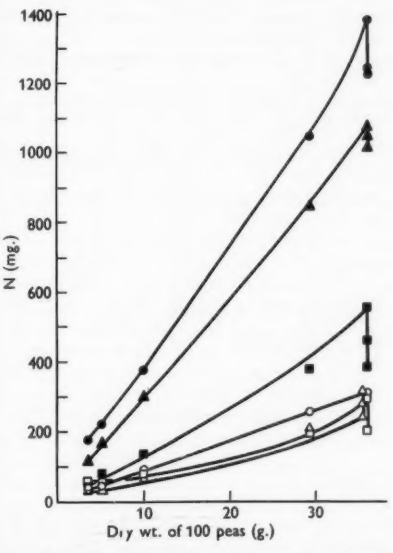


Fig. 1. Nitrogen distribution in extracts of pods at different stages of growth. ●, Total N; ▲, total non-protein N; ■, amino N; ○, protein N; △, peptide N; □, amide N.

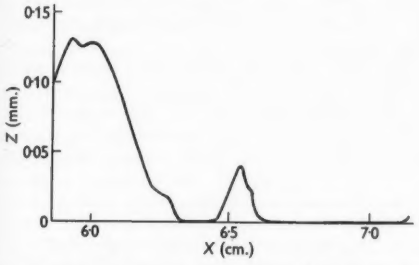


Fig. 2. Typical ultracentrifuge diagram of a protein preparation from pods of *Pisum sativum* (stage V). Time: 20 min., scale distance: 40 mm. The ordinate is proportional to the gradient of refractive index; abscissa is distance from the centre of rotation.

DISCUSSION

As can be seen from Table 3, the decrease in total nitrogen concentration is closely paralleled by a decrease in protein concentration, whereas the concentration of amino, amide and peptide nitrogen remains remarkably constant throughout the ripening process. These data, together with those in Fig. 1, illustrate once more very clearly that protein is first synthesized in the pods and then broken down again and transferred to the developing seeds. It is worth noting, however, that it is not broken down completely to amino acids, but that a considerable portion remains in peptide linkage. As the amino nitrogen comprises the free amino group of asparagine and also the free amino groups of peptides, it can be seen that very little exists in the form of amino acids. Since, however, there is very little amide in the pod protein (about 1·5%) the large amounts of free amide must be formed by secondary reactions from amino acids and carbohydrate residues, as with seedlings (Prianischnikow, 1922).

The question of the role of amide in the transport of nitrogen in the plant has been extensively discussed by Mothes (1926). On the basis of detailed analyses of different anatomical parts of plants this author concluded that amides undoubtedly figure in the translocation of nitrogen. One would expect, however, that not only amide but any low-molecular product present in appreciable concentration would be translocated. The present data would seem to indicate that peptides resulting from proteolysis, as well as amides, are in this category.

Another point brought out in the course of this investigation is the influence of water content in the regulation of protein synthesis. As it has been seen from the results on whole peas, ensuing desiccation brings about important changes in the composition of the seeds, which become apparent in the form of a sharp increase in protein. By examining the data in Fig. 1, it can be seen that at the stage corresponding to ensuing desiccation of the seeds (it should be remembered that pods VI yielded mainly peas of stage V), the breakdown of nitrogenous products in the pods is faster than the synthesis, as can be seen from the sharp breaks in the curves. (The sharp increase in peptide nitrogen in the seeds together with the sharp decrease in protein nitrogen in the pods should be noted.) This is despite the fact that the moisture content of the pods remains remarkably constant throughout (see Table 2), which shows the interdependence of metabolic processes in various organs of the plants, and indicates, as well, the fact that no generalization concerning the influence of water content in the formation of protein can be made. In the seeds, reduction of moisture causes an increase in protein; in the pods protein decreases whereas moisture

remains constant; and in the leaves (Petrie & Wood, 1938) the net rate of protein synthesis increases with increased water content.

SUMMARY

1. Pods of *Pisum sativum* at seven different stages of development have been analysed for total, protein, amino, amide and peptide nitrogen.

2. Protein is first formed, then broken down and transferred to the developing seeds in the form of amide and peptides.

3. The moisture content of the pods has no influence on protein hydrolysis, which, rather, is shown to be determined by the metabolic state of the seeds.

The author wishes to express special thanks to Professor Arne Tiselius, at whose suggestion the present series of investigations was carried out, for his interest and much valuable and friendly advice as well as for the privilege of using the facilities of the Biochemical Institute. Sincere thanks are also due to Laborator Kai O. Pedersen for advice on ultracentrifuging, to Mr Ewald Hellman for the calculations of the ultracentrifuge data, and to Miss Gunnel Englund for the Kjeldahl determinations.

The author is indebted to Professors C. H. Li and E. W. Yemm, F.R.S., and to Dr R. L. M. Synge, F.R.S., for suggestions concerning the manuscripts.

REFERENCES

Bisson, C. S. & Jones, H. A. (1932). *Plant Physiol.* **7**, 91.
Emmerling, A. (1880). *Landw. Versuchsw.* **24**, 113.
Mothes, K. (1926). *Planta*, **1**, 472.
Petrie, A. H. K. & Wood, J. G. (1938). *Ann. Bot., N.S.*, **2**, 33.
Pfenninger, U. (1909). *Ber. dtsch. bot. Ges.* **27**, 227.
Prianischnikow, D. (1922). *Ber. dtsch. bot. Ges.* **40**, 242.
Raacke, I. D. (1957). *Biochem. J.* **66**, 101.
Schulze, E. & Winterstein, E. (1910). *Hoppe-Seyl. Z.* **65**, 431.
Wassilieff, N. (1908). *Ber. dtsch. bot. Ges.* **26**, 454.

The Effect of Parathormone on the ^{32}P Uptake by the Bones of Rabbits and Cats

By J. DAWSON, S. M. WEIDMANN and H. G. JONES
Department of Biochemistry, University of Leeds, and The Biological Research Unit, Dental School and Hospital, University of Leeds

(*Received* 2 *March* 1956)

The two major hypotheses explaining the mechanism of action of parathormone on bones are those of Albright & Ellsworth (1929) and Thompson & Collip (1932). Albright & Ellsworth (1929) suggested that the main action of parathormone is on the kidney, inhibiting the reabsorption of phosphate by the renal tubules. This results in a tendency to a decreased serum inorganic phosphate level, which induces a withdrawal of phosphate from bone to restore the $[Ca] \times [PO_4]$ product in the serum to normal. The hypothesis of Thompson & Collip (1932) postulated a direct demineralizing effect of parathormone on bone. Subsequent investigations by Heller, McLean & Bloom (1950), on histological changes in bone after parathormone treatment, and by Barnicot (1948), on the local demineralization of bone with parathyroid tissue grafted in apposition to it, have increasingly supported the view that parathormone has a direct effect on bone, producing demineralization. Engel (1952) suggested that this demineralization was due to a destruction of osteoid matrix. The effect of parathormone on the renal tubules cannot be excluded, as Harrison & Harrison (1941) and Jacobs & Verbanck (1953) have demonstrated a decreased tubular reabsorption of phosphate in normal animals. Other workers (Dent, 1953; Stoerck & Silber, 1949) found, however, that parathormone had no phosphate diuretic effect in normal subjects but did show this effect in parathyroidectomized preparations. This led to the concept that two parathormones may exist, one of which exerts a maximum effect on bone, whereas the other, by affecting the kidneys, produces a phosphate diuresis.

The ^{32}P uptake by bone could depend on the operation of the following processes: an ion exchange between phosphate ions on the mineral crystal surface and ions in the surrounding fluid, a recrystallization of the bone crystal which would reduce the availability for re-solution of ions already deposited and also active growth and remodelling of bone which would, presumably, depend on cellular activity (Neuman & Weikel, 1954). The distribution of ^{32}P in bones of animals injected with the isotope has been investigated by two different techniques: autoradiography and radioactivity measurements. Autoradiography (Amprino & Engström, 1952) demonstrates in a qualitative way

the uneven distribution of the isotope, whereas with radioactivity measurements of bone extracts the tracer uptake can be determined quantitatively. In recent years various methods have been employed to demonstrate the anatomical distribution of tracers in bone, of which that of Weidmann (1956), with an improved technique of differential extraction, showed the largest regional difference in the magnitude of ^{32}P uptake by shaft bone. The endosteal layer was the most active region, the periosteal layer considerably less active, and the large middle portion of the shaft substantially less than the other two areas. The high activity of the endosteal layer, and to a lesser extent the periosteal layer, might be due to their being areas of active bone formation balanced by active remodelling of bone.

Engfeldt & Zetterström (1954), investigating the activity of parathormone on bone and using the techniques of radioactivity measurements of $(NH_4)_2SO_4$ extracts, autoradiography and microradiography, found that in the young rat parathormone decreased the uptake of ^{32}P into the bones, whereas in older animals the uptake of ^{32}P was increased. Microradiographs, prepared from bones of dogs, showed considerable bone destruction, but a marked increase of ^{32}P was seen with autoradiographs in areas of active bone formation. From this they concluded that the primary action of parathormone is an acceleration of bone destruction in all age groups, leading, eventually, to increased bone formation. This is more apparent in older animals where less new bone formation is occurring. The results of Engfeldt & Zetterström (1954) with the technique of radioactivity measurements of $(NH_4)_2SO_4$ extracts were only average results from bones in which marked regional differences of biological activity exist, as shown by autoradiography.

The present investigation was undertaken to obtain more specific information about the effect of parathormone on different areas of bone. To determine whether the kidneys were involved in any effects observed, some experiments were performed on nephrectomized preparations. By this means, it was thought, conclusions could be reached on the relative effect of ion exchange and active bone formation in connexion with the action of parathormone.

METHODS

Fully grown rabbits and cats were used for all experiments. The effect of parathormone on the turnover of ^{32}P in bones was investigated by the intraperitoneal injection of parathormone (Eli Lilly and Co.). Animals were injected with varying doses for different periods of time. Nine untreated rabbits and six untreated cats were examined some time previously, and these groups served as controls for all further experiments. In nephrectomized animals, maintained under Nembutal anaesthesia, the parathormone injection was made immediately after nephrectomy and the ^{32}P injection 3 hr. later. After another 4 hr. the animal was sacrificed. Control animals were maintained under anaesthesia only (for 3 hr.) before the ^{32}P injection, or were nephrectomized and anaesthetized, but received no parathormone injection. To some nephrectomized preparations massive doses of inorganic phosphate were administered by repeated intravenous injections.

Blood for the serum calcium and inorganic phosphate determinations was obtained by heart puncture from anaesthetized cats and from the marginal vein of the rabbit's ear. Serum-calcium levels were determined by the Clark & Collip (1925) modification of the Kramer–Tisdall method, and serum inorganic phosphate was estimated as described previously (Rogers, Weidmann & Jones, 1953). Samples for bone investigations were prepared by the usual technique (Weidmann & Rogers, 1950; Rogers *et al.* 1953). The specific activity of phosphorus was measured in the cortical and cancellous parts of the shaft bone. In order to obtain the average specific activities of these bone types, representative bone-powder samples were collected from the diaphysis and metaphysis, dissolved in 20% trichloroacetic acid (TCA) and measurements carried out on samples of these solutions. The uptake of ^{32}P was also measured in the subperiosteal, 'middle' and endosteal areas of the diaphysis, from which differential extracts were obtained by the paraffin-protection method. This method and its application have been described elsewhere (Weidmann, 1956). By extracting bone fragments with saturated $(NH_4)_2SO_4$ solutions, with the above method highly active fractions can be obtained which are particularly suitable for study of the changes of phosphorus turnover in animals under pathological conditions. Ammonium sulphate extracts were also prepared from bone-powder samples of metaphyseal trabeculae.

The CO_2-combining power of plasma in the nephrectomized rabbits was measured by the manometric procedure of Van Slyke.

The same amount of tracer was injected into all animals, i.e. 33 μc of carrier-free $H_3{}^{32}PO_4$/kg. body wt. Technique of tracer administration and calculation of specific activity has been described previously (Weidmann, 1956). Radioactivity measurements were always made on animals killed 4 hr. after the administration of the tracer.

RESULTS

Effect of parathormone on the turnover of inorganic phosphorus in various bones and parts of bones of rabbits and cats

Engfeldt & Zetterström (1954) concluded from radioactivity measurements and microradiographic evidence that the primary action of parathormone is an enhanced bone destruction, which eventually leads to increased new bone formation. Since the rate of mineral-salt replacements varies to a large extent within the bone (Weidmann, 1956), the above measurements of radioactivity in $(NH_4)_2SO_4$ extracts from average bone-powder samples are probably not sufficiently specific to establish the

characteristic effects of parathormone action. In the present investigation the effect of parathormone on the specific activity of phosphorus was tested in average samples of cortical and cancellous bone, and in the easily exchangeable fraction of bone salts from various layers of the diaphyses of rabbits and cats.

Table 1 demonstrates the changes observed in serum-calcium and inorganic-phosphorus levels after parathormone treatment. In rabbits the serum values show no significant alteration. In cats treated for a long time with high parathormone dosage the serum-calcium level increased to a mean of 17·5 mg. % as compared with 11·3 mg. % in the control animals, and serum-inorganic phosphorus to a mean of 9·8 from 6·7 mg. % in the normals. Seven hours after a single injection of parathormone neither rabbits nor cats show any significant change in the serum values.

The only change in the specific activity of serum phosphorus, as a consequence of parathormone treatment, was found in the 4 hr. experiment with rabbits [Table 1, column (3)]. The comparatively high concentration of ^{32}P in the serum of these animals may indicate a decrease in the rate of bone-salt deposition from blood, although other factors such as rate of urinary phosphate excretion may play a part.

Rabbit. In Table 2 average specific activities of ^{32}P in bone are given for control animals and for those subjected to parathormone treatment. The values for the three areas of cortical bone represent averages of two different estimations, one for the femur and the other for the tibia. The agreement between the two values was satisfactory.

The effects of parathormone were only shown in short-term experiments after a single injection of 200 U.S.P. units of parathormone. In experiments of longer duration, such as 8 and 20–28 days of parathormone administration, no statistically significant bone changes were observed. In short-term experiments a large reduction in the specific activity of the endosteal layer occurred: as compared with the control value (t 2·2, $P<0·05$; Table 2, column 4). The ratio between the inner and middle layers was not significantly reduced (25·0–16·5). No effect of parathormone is observed on the sub-periosteal layer. No changes were observed in the specific activity of the cancellous bone either. There are, apparently, differences in the mechanism of calcification between the two bone types, and the one operating in the cancellous bones is perhaps less affected by parathormone.

Cat. The cat appears to be a much more suitable animal in which to examine the long-term effects of parathormone than the rabbit. Whereas there was no deterioration in the general health of the rabbit, most cats became severely ill after a few days of hormone administration. This is an interesting difference in the susceptibility of the two species to parathormone. Several animals lost weight considerably, two died, and others were on the brink of death after 7 days of treatment. A diminution of dosage from 200 to 100 U.S.P. units/day decreased the mortality, but the ill-effect was still noticeable, particularly in the youngest of the mature animals.

The cat differs from the rabbit in that the effect is only shown after some days of hormone administration (Table 3). After 9 days the specific activity in

Table 1. *Effect of parathormone on the percentage concentration of inorganic calcium and phosphorus, and the specific activity of serum in rabbits and cats, measured 4 hr. after the injection of* ^{32}P

For definition of specific activities see Methods. In columns (1) and (2) mean values and ranges are given.

Expt.	No. of animals	Ca (mg. %) (1)	P (mg. %) (2)	Specific activity (Mean ± S.E.M.) (3)
		Rabbit		
Control animals	9	13·1 11·1–14·1	5·4 3·4–7·2	20 300 ± 2230
200 U.S.P. units daily for 28 days	6	13·8 13·1–14·5	4·9 4·3–5·5	22 030 ± 670
100 U.S.P. units daily for 8 days	6	12·6 12·3–12·9	5·5 3·1–8·4	25 800 ± 980
200 U.S.P. units, single injection	4	12·3 11·5–13·5	5·8 3·5–7·8	37 080 ± 1550
		Cat		
Control animals	6	11·3 9·4–12·5	6·7 5·4–9·5	33 600 ± 3080
100 U.S.P. units daily for 9 days	5	17·5 13·8–23·9	9·8 5·6–22	29 860 ± 2840
200 U.S.P. units, single injection	4	11·4 10·3–12·4	6·3 5·8–7·2	29 270 ± 2575

Table 2. *Effect of parathormone on the specific activities of phosphorus in bones and parts of bones of rabbits, measured 4 hr. after the injection of* ^{32}P

Results represent mean specific activities ± S.E.M. For the definition of specific activities see Methods.

		Cortical bone						Cancellous bone	
			$(NH_4)_2SO_4$ extract of layers of bone shaft			Ratio of specific activity			
Expt.	No. of animals	TCA extract of shaft (average) (1)	Outer (2)	Middle (3)	Inner (4)	Outer / Middle (5)	Inner / Middle (6)	TCA extract of metaphysis (average) (7)	$(NH_4)_2SO_4$ extract of bone powder from metaphysis (8)
Control animals	9	46 ± 6·0	656 ± 133	149 ± 30	3910 ± 620	4·7 ± 0·7	25·0 ± 3·9	336 ± 53	2100 ± 570
200 U.S.P. units daily for 28 days	6	57 ± 7·7	637 ± 83	129 ± 26	3650 ± 579	6·7 ± 2·8	32·4 ± 4·0	439 ± 58	2125 ± 670
100 U.S.P. units daily for 8 days	6	53 ± 3·7	934 ± 115	204 ± 12	5780 ± 990	4·7 ± 0·7	28·5 ± 5·6	415 ± 47	3390 ± 687
200 U.S.P. units, single injection	4	44 ± 5·0	448 ± 29	118 ± 17	1820 ± 230	4·1 ± 0·6	16·5 ± 2·9	380 ± 44	1996 ± 422

Table 3. *Effect of parathormone on the specific activities of phosphorus in bones and parts of bones of cats, measured 4 hr. after the administration of* ^{32}P

Results represent mean values ± S.E.M. For the definition of specific activities see Methods.

		Cortical bone						Cancellous bone	
			$(NH_4)_2SO_4$ extract of layers of bone shaft			Ratio of specific activity			
Expt.	No. of animals	TCA extract of shaft (average)	Outer	Middle	Inner	Outer / Middle	Inner / Middle	TCA extract of metaphysis (average)	$(NH_4)_2SO_4$ extract of bone powder from metaphysis
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Control animals	6	19·0 ± 2·6	477 ± 70	80 ± 14	2180 ± 313	5·8 ± 0·8	31·3 ± 5·1	273 ± 86	2160 ± 309
100 U.S.P. units daily for 9 days	5	26·3 ± 3·3	431 ± 68	63 ± 9	959 ± 87	6·9 ± 1·0	16·6 ± 4·0	392 ± 88	—
200 U.S.P. units, single injection	4	31·0 ± 9·0	500 ± 156	72 ± 16	1750 ± 710	7·0 ± 0·8	22·4 ± 1·1	348 ± 96	1683 ± 338

the endosteum is markedly reduced as compared with the control group (t 3·4, $P<0·01$; Table 3, column 4), whereas the activity of the sub-periosteum is not affected. The ratio between the endosteum and the middle layer fell from 31·3 to 16·6. In both species the overall specific activities of the shaft are not altered after parathormone administration; the effect seems to be restricted to the endosteum. The cancellous bones, like those of the rabbit, showed no sign of parathormone influence.

Effect of parathormone in nephrectomized animals

According to Albright & Ellsworth (1929) the increase in bone resorption during hyperparathyroidism is due to a lowering of the serum-phosphate level owing to phosphate diuresis. In order to test this hypothesis the kidneys were ligated and the action of parathormone was examined under these conditions (Table 4).

Since nephrectomized animals were maintained under Nembutal anaesthesia for the whole experiment, the effect of anaesthesia on the ^{32}P turnover of bone was first investigated. Anaesthesia has no effect on the overall uptake of tracer into the cortical part of the diaphysis, whereas nephrectomy causes a marked reduction (t 2·6, $P<0·05$; Table 4, column 1), which is due to a decrease in endosteal activity. Anaesthesia and nephrectomy together decrease the ^{32}P-uptake of the endosteal layer, whereas anaesthesia alone does not affect it. A comparison between the two groups shows a significant decrease in the former preparation (t 2·8, $P<0·05$). The activity in the middle layer is not significantly decreased in either nephrectomized or anaesthetized preparations (t 2·15, P 0·05 and t 1·5, $P<0·2$ respectively; Table 4, column 3). The ratio of the ^{32}P-uptake into the inner and middle areas is significantly increased above control values in both the anaesthetized and anaesthetized nephrectomized preparations (t 3·8, $P<0·01$ and t 2·5, $P<0·05$ respectively; Table 4, column 6).

The decrease in specific activity may be due to increased bone resorption, or to a decreased rate of equilibration with ^{32}P in the blood, in which the serum-calcium level has increased from 12·8 to 20·8 mg. % (mean values), without alteration in the serum inorganic phosphorus concentrations. An increase in the serum-calcium concentration, together with an increased bone resorption, could be caused by development of massive acidosis, but this would not explain the constant phosphorus concentrations in the serum. Acidosis is, however, not likely to have developed, because the blood CO_2-combining power of these animals is normal. In nephrectomy the activity of the endosteal layer is decreased relative to the values found for anaesthetized preparations. Nephrectomy can there-

Table 4. *Effect of anaesthesia, nephrectomy, parathormone and phosphate infusion on the specific activity of phosphorus in bones and parts of bones of rabbits, measured 4 hr. after the administration of* ^{32}P

Results represent specific activities ± S.E.M. For the definition of specific activities see Methods.

		Cortical bone						Cancellous bone	
		TCA extract of shaft (average)	$(NH_4)_2SO_4$ extract of layers of bone shaft			Ratio of specific activity		TCA extract of metaphysis (average)	$(NH_4)_2SO_4$ extract of bone powder from metaphysis
Expt.	No. of animals	(1)	Outer (2)	Middle (3)	Inner (4)	Outer Middle (5)	Inner Middle (6)	(7)	(8)
Control animals	9	46 ± 6·0	656 ± 133	149 ± 30	3910 ± 620	4·7 ± 0·7	25·0 ± 3·9	336 ± 53	2100 ± 570
Anaesthesia	3	41 ± 0·2	483 ± 10	72 ± 14	4071 ± 535	7·6 ± 1·4	65·6 ± 16·5	—	—
Anaesthesia and nephrectomy	7	27 ± 3·4	367 ± 47	71 ± 15	2720 ± 208	5·6 ± 1·5	41·6 ± 5·4	231 ± 26	1150 ± 173
Anaesthesia, nephrectomy and parathormone	3	16 ± 1·9	255 ± 11	75 ± 24	985 ± 80	3·6 ± 1·2	17·3 ± 4·6	183 ± 32	1050 ± 203
Anaesthesia, nephrectomy and PO_4 infusion	7	109 ± 23	1991 ± 408	236 ± 43	4810 ± 1060	8·1 ± 0·9	20·4 ± 3·1	626 ± 93	2571 ± 350

fore affect the activity of the endosteal layer, by some mechanism other than production of acidosis.

Effect of parathormone on nephrectomized rabbits

The effect of parathormone was tested on nephrectomized rabbits only, since the cat does not respond to parathormone action in the short time interval (7 hr.) available for these experiments, whereas in the rabbit immediate responses were seen to parathormone injection.

The effect of parathormone on nephrectomized rabbits was tested by injecting 200 U.S.P. units of parathyroid extract immediately after nephrectomy. The results are contained in Table 4. There is no significant decrease of specific activity in whole-bone samples from cortical and cancellous bones (t 2·0, $P<0·1$ and t 1·1, $P<0·3$ respectively; Table 4, columns 1 and 7). The outstanding effect of parathormone on nephrectomized rabbits appears to be the big fall in the specific activity of phosphorus in the endosteum (t 5·17, $P<0·001$; Table 4, column 4). Apart from this difference, there is also a drop in the endosteal activity in relation to the middle area when anaesthetized and nephrectomized preparations are compared with similar animals treated with parathormone (t 2·71, $P<0·05$). Since in intact and nephrectomized animals treated with parathormone a decreased ratio of specific activities of endosteal:middle layer is observed, it seems likely that this is a characteristic of parathormone activity and is, presumably, caused by a direct effect on bone. The kidneys were eliminated in this experimental set-up, consequently the reduction in the endosteal area cannot be initiated by a primary lesion of the kidneys.

It has been suggested that in nephrectomized animals there is a rise of serum inorganic phosphorus (which was found only in cats in these experiments), and that this rise may act as a stimulus to parathyroid activity (Crawford, Osborne, Talbot, Terry & Morrill (1950)). Varying amounts of sodium phosphate buffer, pH 7·4 (120–420 mg. of P), were injected into nephrectomized rabbits, to investigate whether increases in serum inorganic phosphorus levels would result in a further decrease in the turnover rate of the inner layer. The results (Table 4) show that phosphate injection considerably increased the magnitude of ^{32}P uptake of the whole bone. This would be expected if precipitation was occurring in the bone without any biological activity being responsible. The interesting feature is that the deposition in the endosteal layer is less than in the other two. This results in the ratio of the specific activities of the endosteal and middle layers being reduced as compared with the nephrectomized preparation (t 3·4, $P<0·01$), which would support the hypothesis that the serum inorganic phosphorus level can act as a stimulus to increased parathyroid activity.

DISCUSSION

The results presented show that in both the cat and the rabbit there is no effect on the ^{32}P uptake in whole-bone determinations, when the animals are treated with either single or multiple doses of parathormone. Lack of effect of parathormone on the renewal rates of bone phosphate in whole bones was also observed by Tweedy & Campbell (1944) in rats. If, however, different parts of the shaft of cortical bone are analysed, a significant reduction is observed in the activity of the endosteal layer of the cat after multiple dosage, and in that of the rabbit after a single injection. It may be significant in this connexion that in the cat multiple dosage produces a rise in serum-calcium level, an effect typical for parathormone treatment. This effect is not observed in the rabbit, which appears more resistant to multiple dosage with parathormone. It would appear that only the more active areas of bone are susceptible to parathormone action, whereas in whole bones the effect is masked by the large areas of comparatively inactive bone. This finding seems to be in agreement with that of Engfeldt & Zetterström (1954), who found a decrease of ^{32}P uptake in the biologically active diaphyses and epiphyses of young rats, and an increase in the more static bones of mature specimens. In cancellous bones no alteration in the ^{32}P uptake due to parathormone was detected, and in this respect the present results differ from those of Engfeldt & Zetterström (1954). The effect of parathormone may be antagonized by strains and stresses in bone tending to increase osteoblastic activity, which may be more pronounced in cancellous than in cortical bones.

In cats an absolute decrease in ^{32}P uptake into the endosteal layer was detected in spite of the absence of the expected decrease in serum phosphate after parathormone administration. It was also shown that a rise in serum-phosphate level in rabbits, brought about by phosphate infusion, caused an absolute increase in endosteal activity, as would be expected if the solubility-product principle operated to produce increased bone-salt precipitation. Therefore the decrease of turnover rate in the endosteal layer is not related to changes in the inorganic-phosphate level, but is a direct effect of parathormone. Stewart & Bowen (1951), using the injection of oxalate to lower the serum-calcium-ion level and thus affect the ionic equilibrium between blood and bone, also found that calcium could only be withdrawn from bone if the parathyroids were intact. This is further evidence that parathormone has a direct effect on bone.

When ^{32}P is incorporated into bone it becomes diluted by a large mass of ^{31}P already present; tracer losses due to bone resorption are therefore not noticeable. The relatively low ^{32}P concentrations found in the endosteum of animals treated with parathormone are more likely to be due to a reduction in ^{32}P-uptake than to losses through bone resorption. It is suggested therefore that the decrease in the specific activity of the endosteal layer is due to a decreased rate of ^{32}P incorporation and not to an increased withdrawal from bone. The injection of parathormone into anaesthetized nephrectomized rabbits demonstrates that the main effect of parathormone on the bones is on the endosteal layer, a finding similar to that seen with parathormone injections in intact animals. The injection of a buffered phosphate solution into a nephrectomized rabbit produces a marked decrease in the serum-calcium level and an increase in the serum inorganic phosphate level. The overall uptake of ^{32}P by the whole bone is increased, as would be expected from an increased deposition of tricalcium phosphate, owing to the markedly increased serum inorganic phosphate level. There is a substantially increased uptake by all three layers but the increase is much smaller in the inner layer. The ratio between the middle and inner layers is therefore reduced, an effect which is also seen with parathormone. That the phosphate effect could be due to a decreased availability of the inner layer for precipitation as compared with the middle layer is unlikely, as the blood supply to the inner layer is likely to be at least as good as that to the middle layer. The decreased serum-calcium level, or the increased serum inorganic phosphate level, may have stimulated the animals' parathyroids. This would result in an inhibition of osteoblastic activity, an effect mainly seen in the inner layer, leading to the relatively smaller ^{32}P uptake in this area. Similarly, nephrectomy alone will decrease the activity of the inner layer which may also be due to a stimulation of the animals' own parathyroids.

Whilst there seems no reason to dispute that exchange and recrystallization of bone minerals may play some part in the maintenance of normal adult-bone structure, it seems likely that none of the effects of parathormone on the uptake of ^{32}P by bone can be explained on this basis. In their histological investigation Heller *et al.* (1950) observed a marked decline in the number of osteoblasts after parathormone treatment, and also a marked increase in osteoclasts. They suggested that osteoclasts were the same cells as osteoblasts but in a different functional state. The present results are interpreted as indicating that the primary effect of parathormone is an inhibition of osteoblastic activity. This decreases the ^{32}P-uptake into the area of bone where osteoblastic activity is maximal, namely the endosteal layer. Some of the osteoblasts become degenerate and others become osteoclasts. These cells are either non-functioning osteoblasts or are cells whose synthetic ability has been inhibited and which now demonstrate their catabolic function in the dissolution of the bone matrix.

SUMMARY

1. The major effect of parathormone on bones in both cats and rabbits is to decrease the uptake of phosphates into the endosteal layer of the diaphyseal shaft, as shown by tracer experiments with ^{32}P.

2. This effect of parathormone can be observed in nephrectomized rabbits.

3. The relation of parathormone to active processes maintaining bone structure is discussed.

We would like to acknowledge the technical assistance given to us by Miss E. B. Morley and Mr R. W. Lofthouse.

REFERENCES

Albright, F. & Ellsworth, R. (1929). *J. clin. Invest.* **7**, 183.
Amprino, R. & Engström, A. (1952). *Acta anat.* **15**, 1.
Barnicot, N. A. (1948). *J. anat.* **82**, 233.
Clark, E. B. & Collip, J. B. (1925). *J. biol. Chem.* **63**, 461.
Crawford, J. D., Osborne, M. M., Talbot, N. B., Terry, M. L. & Morrill, M. F. (1950). *J. clin. Invest.* **29**, 1448.
Dent, C. E. (1953). *Proc. R. Soc. Med.* **46**, 291.
Engel, M. B. (1952). *Arch. Path.* **53**, 339.
Engfeldt, B. & Zetterström, R. (1954). *Endocrinology*, **54**, 506.
Harrison, H. E. & Harrison, H. C. (1941). *J. clin. Invest.* **20**, 47.
Heller, M., McLean, F. C. & Bloom, W. (1950). *Amer. J. Anat.* **87**, 315.
Jacobs, E. & Verbanck, M. (1953). *Acta med. scand.* **145**, 143.
Neuman, W. F. & Weikel, J. H. (1954). *Atomic Energy Report*, UR-314.
Rogers, H. J., Weidmann, S. M. & Jones, G. H. (1953). *Biochem. J.* **54**, 37.
Stewart, G. S. & Bowen, H. F. (1951). *Endocrinology*, **48**, 568.
Stoerck, H. C. & Silber, R. H. (1949). *Fed. Proc.* **8**, 371.
Thompson, D. L. & Collip, J. B. (1932). *Physiol. Rev.* **12**, 309.
Tweedy, W. R. & Campbell, W. W. (1944). *J. biol. Chem.* **154**, 339.
Weidmann, S. M. (1956). *Biochem. J.* **62**, 593.
Weidmann, S. M. & Rogers, H. J. (1950). *Biochem. J.* **47**, 493.

The Effect of X-Radiation *in vivo* on Protein and Nucleoprotein Metabolism in the Rat

By J. E. RICHMOND,* MARGERY G. ORD and L. A. STOCKEN
Department of Biochemistry, University of Oxford

(*Received* 17 *September* 1956)

A satisfactory explanation is not yet available of the difference in sensitivity of various animal tissues to ionizing radiations. The rule of Bergonie & Tribondeau (1906) relates sensitivity to mitotic activity, and in general it is obeyed, but the behaviour of the lymphocytes is anomalous and that of thymus questionable. We have considered the possibility (Ord & Stocken, 1955, 1956*a*) that the damage may depend on the relative volumes and metabolic activities of the cytoplasm and the nucleus; similar ideas have been expressed by Schjeide, Mead & Myers (1956). It is, however, not easy to design unequivocal experiments to decide this point, and we have therefore concerned ourselves with a comparison of the effect of X-radiation on the incorporation of phosphate, glycine and lysine into subcellular fractions of several rat tissues.

Some studies have been carried out on the incorporation of amino acids into nucleoprotein (Allfrey, Daly & Mirsky, 1955*a*), but the effect of radiation *in vivo* on the incorporation has not been examined nor has a comparison been made of the simultaneous incorporation of phosphate into nucleic acid and an amino acid into the histone. We therefore decided to investigate this in a radiosensitive and non-sensitive tissue and chose thymus and liver as examples.

Since comparatively few studies have been made of protein metabolism in several organs in the same animal after irradiation, the experiments were extended to include a comparison of the rates of incorporation of lysine and glycine into the proteins of spleen, pancreas and bone marrow, which differ in their radiosensitivity and their capacity to form proteins. Such an investigation might give some indications as to the site or sites of the radiation lesion in the cell and perhaps allow a correlation of metabolic disturbance with the radiosensitivity of the various organs.

METHODS

Animals. Eight male litter-mates of the laboratory strain of Wistar rats weighing approx. 75 g. were used in each experiment. Four of the animals served as controls. All rats were fasted for 2·5 hr. before administration of the isotopes, and were killed by decapitation 2 hr. afterwards.

Irradiation. X-Radiation was carried out *in vivo* at the M.R.C. Radiobiological Research Unit, Harwell. The characteristics of the radiation were: half-value layer 2·5 mm. Cu, peak voltage 250 kv. at 14 ma. The rats received 1000 r. total body radiation in 14 min. 38 sec.

Administration of labelled isotopes

Phosphate. Carrier-free sodium [^{32}P] phosphate was obtained from The Radiochemical Centre, Amersham, and was brought to pH 7 before use. The animals were injected intramuscularly with 50 μc/100 g. body wt. immediately after irradiation.

Amino acids. An enzymic hydrolysate of casein was obtained from Benger's Ltd., Holmes Chapel, Cheshire. [2-^{14}C]Glycine was purchased from The Radiochemical Centre, Amersham. Uniformly labelled L-[^{14}C]lysine was prepared by growing *Chlorella* in a $^{14}CO_2$ atmosphere as described by Dutton (1956), and the lysine isolated from an enzymic hydrolysate by the chromatographic method of Hirs, Moore & Stein (1952). A solution was made to contain 800 mg. of casein hydrolysate, 15 mg. of cysteine and 195 μc of glycine (specific activity 50 μc/mg.) in 5 ml.; 20 μc/100 g. body wt. was injected intraperitoneally immediately after the injection of ^{32}P. Labelled lysine was substituted for labelled glycine in the second experiment.

Preparation of samples. Expt. 1

Two hours after administering the isotopes, the animals were decapitated and the blood was collected in heparinized dishes. The plasma was separated and the specific activities of the inorganic phosphate and plasma proteins were determined as a reference for the incorporation experiments. Pancreas, liver, spleen and the long bones were quickly excised and frozen in liquid oxygen.

Thymus. The whole thymus was taken and deoxyribonucleic acid nucleoprotein prepared as described by Crampton, Lipshitz & Chargaff (1954). The nucleoprotein was caused to dissociate by 2·6 M-NaCl and the deoxyribonucleic acid (DNA) removed by precipitation with ethanol. After deproteinizing twice more with sodium dodecyl sulphate (Chargaff, 1955) the DNA was extracted as described by Deluca, Rossiter & Strickland (1953). Ion-exchange chromatography was used to separate ^{32}P-labelled deoxyribonucleotides and paper electrophoresis for the ^{14}C-labelled nucleotides. In both cases about 80% of the radioactivity was recovered and no evidence could be obtained of non-nucleotide contamination.

The supernatant from the first deproteinization was dried in a frozen state, redissolved in water and, after dialysis,

* Commonwealth Fund Fellow, New York.

treated by the method of Daly & Mirsky (1955) for the isolation of the histone fractions.

Liver, pancreas and spleen. Nuclei were prepared by the method of Dounce, Witter, Monty, Pate & Cottone (1955) and the nucleoprotein as well as DNA were isolated according to the procedure of Crampton *et al.* (1954). The histone fraction was obtained by the modified method of Daly & Mirsky (1955) as in the experiment with thymus, except that it was not fractionated. Mitochondrial and microsomal fractions were obtained by the procedure of Dounce *et al.* (1955) and the proteins were prepared, dialysed and analysed by the methods of Littlefield, Keller, Gross & Zamecnik (1955).

Blood and bone marrow. Haemoglobin was prepared from washed cells by the procedure of Drabkin (1949). Plasma proteins were prepared by the methods of Miller, Bly, Watson & Bale (1951). Bone-marrow proteins were obtained from a bone-marrow supernatant (Richmond, Altman & Salomon, 1951) by precipitation with 2·8M phosphate buffer, pH 6·8 (Drabkin, 1949). The precipitate was washed exhaustively with ethanol–ether–chloroform and was found to be about 90% haemoglobin on the basis of the haematin content.

Preparation of samples. Expt. 2

In the second experiment the liver, as well as the thymus, was processed without delay. The methods were the same as in the first experiment except that the medium of Barnes, Esnouf & Stocken (1956) was used in the isolation of nuclei, mitochondria and microsomes. Thymus deoxynucleoprotein was obtained from the nuclei as in Expt. 1, and the histones were liberated as before. The incompletely deproteinized DNA obtained at the NaCl–ethanol stage was then shaken overnight in water, pH 7·0, at 2°, and the insoluble residue separated to give the residual DNA–protein fraction. The DNA was deproteinized, extracted and estimated as in Expt. 1. Spleen and bone marrow were not investigated.

Measurement of radioactivity

The ^{32}P activity was measured as previously described (Ord & Stocken, 1956*a*). For the ^{14}C measurements samples of the order of 500μg. were weighed on aluminium planchettes 1·5 cm. in diameter and spread over the entire surface with solvent. The dried samples were then counted in a helium gas-flow counter as described by Garrow & Piper (1955). A background count was determined at least twice each day and the plateau for this counter was checked at least once each day with the use of a standard. The efficiency of the counter was approximately 35%. Sufficient counts were recorded to give a counting error of less than 3%. Duplicate samples gave reproducible counting rates within 5%.

RESULTS

The simultaneous uptake of phosphate and of amino acid into nuclear material has been studied with two amino acids which have different anabolic fates. Glycine was used since it takes part both in DNA and in protein synthesis; lysine on the other hand is metabolically less versatile and does not find its way into DNA.

^{32}P phosphate and [2-^{14}C]glycine or [^{14}C]lysine were injected and the control and irradiated animals killed 2 hr. later. The specific activity of the phosphorus in DNA was referred to that of plasma inorganic phosphate but since we had no similar standard for the amino acids and since there was no difference in the specific activities of the plasma proteins of normal and irradiated rats the results were expressed as counts/min./mg. of material.

The inhibition of ^{32}P incorporation into thymus DNA (Table 1) was the same as that previously obtained under the same conditions (Ord & Stocken, 1956*a*), and the reduction in glycine uptake confirms the earlier experiments of Harrington & Lavik (1955), who showed that 100 r. X-radiation lowered the incorporation of formate 24 hr. after exposure. The inhibition seemed to be rather greater with glycine than with phosphorus but more evidence would be required to substantiate this.

The data in Table 1 indicate that all thymus nuclear fractions investigated were adversely affected by irradiation, and there was little difference in the response between the fractions. Mitochondria and microsomal supernatant (soluble) proteins were not altered by irradiation, while there was a slight decrease in the incorporation of lysine into the microsomal-protein fraction. It is interesting to note that the histone fractions of thymus had the highest specific activity of all the fractions studied in this tissue at this time interval. In the experiments with radioactive glycine, the 'arginine-rich', 'lysine-rich' and the histone fraction precipitated with saturated NaCl had about the same specific radioactivities, although their glycine contents are different (Allfrey *et al.* 1955*a*).

Table 1. *Effect of X-radiation on the incorporation of carbon and phosphorus precursors into fractions obtained from rat thymus*

All results are expressed as means ± standard deviation. Each group consisted of four animals.

Fraction	Control	Irradiated
Glycine incorporation (counts/min./mg.)		
DNA	376±8	91±11
Total histone	473±33	247±17
'Arginine rich' histone	459±31	217±2
'Lysine rich' histone	487±40	276±31
Acid-insoluble residue of histone fraction	227	106
Lysine incorporation (counts/min./mg.)		
Total histone	2064±242	1342±67
Residue DNA–protein	285±57	154±23
Mitochondria	1153±110	1128±34
Microsomes	771±160	598±46
Microsomal supernatant	1629±115	1629±50
Phosphorus incorporation (relative to specific activity of plasma inorganic P × 10^2)		
DNA (glycine experiment)	4·5±0·2	1·8±0·2
DNA (lysine experiment)	3·2±0·7	1·1±0·4

Similarly, under the conditions of our experiments the activity of the total histones of liver (Table 2) was always greater than that of the residual protein. Allfrey *et al.* (1955*a*), however, using [^{15}N]glycine under different conditions, found that incorporation *in vivo* into liver, pancreas and kidney nucleohistones of mice was very low compared with that into the residual protein fraction. The ^{14}C activity of our rat-liver histones after administration of glycine was higher than that of thymus; with lysine little difference was detectable. Amino acid uptake into the liver nuclear fractions was if anything slightly increased, as has been reported for acetate incorporation into liver protein by Hevesy (1949*a*) and for phenylalanine into a number of liver-protein fractions by Butler, Cohn & Crathorn (1956). Glycine incorporation into DNA was unaffected although ^{32}P uptake was reduced.

Table 2. *Effect of X-radiation on the incorporation of carbon and phosphorus precursors into fractions obtained from rat liver*

Results are mean values of four animals ± S.D.

Fraction	Control	Irradiated
Glycine incorporation (counts/min./mg.)		
DNA	131 ± 22	152 ± 46
Total histone	995 ± 175	1278 ± 154
Acid-insoluble residue of histone fraction	547 ± 88	629 ± 72
Mitochondria	984 ± 115	945 ± 63
Microsomes	966 ± 130	1022 ± 110
Microsomal supernatant	743 ± 80	903 ± 105
Deoxycholate-soluble portion of microsomes	1082 ± 74	1229 ± 100
Deoxycholate-insoluble portion of microsomes	638 ± 63	737 ± 105
Lysine incorporation (counts/min./mg.)		
Total histone	1544 ± 127	1769 ± 130
Residue DNA–protein	723 ± 85	760 ± 62
Mitochondria	941 ± 65	1076 ± 69
Microsomes	581 ± 90	761 ± 140
Microsomal supernatant	1453 ± 148	1749 ± 81
Phosphorus incorporation (relative to specific activity of plasma inorganic P × 10^2)		
DNA (lysine experiment)	0·73 ± 0·09	0·38 ± 0·18

Amino acid incorporation into the proteins of pancreas (Table 3) was more variable but did not show any striking changes after irradiation. The soluble proteins had the highest observed specific activity of this organ as well as of the entire animal.

Table 4 contains the data on blood and bone marrow. The incorporation of amino acids into the plasma proteins and circulating haemoglobin was not measurably altered by irradiation. Fractionation of the plasma proteins failed to show a radiation lesion. There was little change in the amount of glycine incorporated into bone-marrow proteins after irradiation.

DISCUSSION

It is conceivable in work involving the disruption of cells followed by the isolation of subcellular fractions that these operations in themselves induce a distribution of the cellular components different from that of the intact cell (Dounce, 1955; Allfrey, Mirsky & Stern, 1955*c*), and this possibility cannot be ruled out in the present work. However, several deductions may be made from the results. In the thymus, the histone fraction contained more radioactivity than any other fraction. The radioactivity of this fraction could only be diluted by other

Table 3. *Effect of X-radiation on the incorporation of amino acids into rat pancreas*

Results are mean values of four animals ± S.D.

	Glycine incorporation (counts/min./mg.)		Lysine incorporation (counts/min./mg.)	
Fraction	Control	Irradiated	Control	Irradiated
Histone	576 ± 72	450 ± 45	220	113
Mitochondria	1728 ± 128	1711 ± 301	1579 ± 265	1596 ± 380
Microsomes	1438 ± 60	1686 ± 300	1235 ± 195	1224 ± 335
Microsomal supernatant	3034 ± 390	3236 ± 356	10310 ± 1400	10265 ± 819

Table 4. *Effect of X-radiation on the incorporation of amino acids into rat haemoglobin and plasma proteins*

Results are mean values of four animals ± S.D.

Substance	Control	Irradiated
Glycine incorporation (counts/min./mg.)		
Haemoglobin, blood	163 ± 15	157 ± 34
Bone-marrow proteins (haemoglobin)	1685 ± 100	1476 ± 100
Total plasma proteins	667 ± 69	686 ± 31
Lysine incorporation (counts/min./mg.)		
Total plasma proteins	2016 ± 148	2324 ± 262

fractions, unless of course there is selective absorption of a highly active fraction on the deoxyribonucleic acid–protein with similar chemical properties and the same solubility characteristics as the deoxyribonucleoprotein and the derived histones. If dissociation followed by re-association with non-specific proteins occurred in the process of isolation, the data obtained indicate that this process is restricted to intranuclear interactions and the effects observed after irradiation should be real unless irradiation specifically induces adsorption.

Protein synthesis as well as deoxyribonucleic acid synthesis, in thymus nuclei seems to be inhibited by radiation. The latter finding has been reported earlier (Hevesy, 1949*a*), as has the inhibition of ^{32}P phosphate uptake into nuclear ribonucleic acid (RNA) (Ord & Stocken, 1956*b*). Even though fractions isolated from thymus nuclei, in addition to histone, exhibited a depression in amino acid incorporation after irradiation, the possibility of these ill-defined proteins being mixtures of denatured histones has not been eliminated. Alternatively, the synthesis of these fractions may be directly related to (Allfrey, Mirsky & Osawa, 1955*b*), or a resultant of, histone formation. Butler *et al.* (1956) have reported very recently that the incorporation of amino acids into total thymus proteins is not altered after 500 r. The latter observation of these investigators is not in disagreement with the present work inasmuch as the histone fractions are not the greater part of the proteins of thymus, and although there is a lowered uptake of ^{32}P into cytoplasmic RNA incorporation into mitochondrial and soluble proteins is unaffected.

Several investigators have studied the incorporation of isotopic amino acids into the proteins of the subcellular fractions of liver from adult animals (Brunish & Luck, 1952; Allfrey *et al.* 1955*a*). The rate of incorporation into the histone fraction, by comparison with other fractions, is faster in the present experiment than has been reported previously. This difference is perhaps due to the greater incorporation in growing rats. It is noteworthy that 2 hr. after administering the amino acids, the histone fraction had about the same level of radioactivity as the soluble proteins. Since only one time period was done, no inference can be made as to the likelihood of some of these fractions having a precursor relationship. Fractionation of the deoxycholate-soluble and -insoluble portions showed that the soluble fractions contained more of the radioactivity than the insoluble fraction, which was about the same in the control and irradiated groups.

An increase in the incorporation of protein precursors into liver proteins after irradiation has been observed previously by Hevesy (1949*b*) and by Butler *et al.* (1956). A very slightly increased incorporation into the fractions was found in the present experiments, with the exception, perhaps, of the nuclear and mitochondrial fractions.

The results for ^{32}P and glycine incorporation into DNA had a wider scatter than those for thymus but there appears to be some difference in the effects of radiation on the uptake of these tracers into liver DNA. Although ^{32}P uptake into DNA is reduced 2 hr. after 1000 r., incorporation into nuclear and cytoplasmic RNAs is unaffected or slightly increased (Ord & Stocken, 1956*a*; Sherman & Almeida, 1956), and glycine uptake was unaffected. Similar discrepancies have been reported in the inhibition of the uptake of various carbon precursors into DNA of rat thymus 24 hr. after 100 r. (Harrington & Lavik, 1955). The reason for these inconsistences is at present unknown, but it is possible that there is some contamination of the DNA with radioactive protein. Recoveries of ^{14}C in nucleotides of thymus DNA seemed to exclude this explanation, but with liver lack of material did not allow similar experiments to be performed. The expected contamination after detergent deproteinization does not usually exceed 0·5% (Frick, 1954), but since in addition to this we subsequently carried out the Hammarsten-extraction procedure it seems unlikely that the specific activity of the DNA can be greatly in error.

Amino acid incorporation into the plasma proteins followed the same general pattern as liver proteins in the irradiated and control groups in confirmation of the observation of Miller (private communication), and similar results were obtained for pancreas. The fluctuation of the levels of radioactivity in the fractions isolated from pancreas is perhaps a resultant of varying degrees of starvation and stimulation of the gland in response to feeding. These results show that pancreas has the capacity to form considerable quantities of proteins, as has been observed by others isolating distinct proteins rather than mixtures of fractions (Hokin, 1956; Daly, Allfrey & Mirsky, 1955). It has been shown recently that amylase synthesis is augmented by radiation (Hokin & Hokin, 1956).

The specific activity of the circulating haemoglobin was about the same in both groups. However, there was not a marked decrease in the amount of amino acids incorporated into the proteins precipitated from a bone-marrow supernatant with 2·8 M-phosphate buffer.

Results obtained for spleen were qualitatively similar to those of liver and pancreas after irradiation. Because of the marked changes in spleen during the first day after irradiation (Kallman & Kohn, 1955) it is difficult to interpret the data procured with this organ. Work is in progress in this laboratory to characterize some of the changes that occur in this organ at short time intervals after irradiation.

The biochemical difference in radiosensitivity of rat tissues is well illustrated when the effects of X-radiation on incorporation of amino acids into DNA and the histone fractions of liver and thymus are considered. In thymus, inhibition of glycine uptake into DNA is accompanied by a marked reduction in glycine and lysine incorporation into the histones, whereas in liver there is little change, or even an enhancement of incorporation into the nucleoproteins. The disturbance in nucleic acid and histone metabolism shown by thymus after exposure, at the same time that certain highly organized, energy-dependent cytoplasmic functions are undisturbed, contributes to the suggestion that the nucleus is the prime site of the metabolic lesion produced by X-radiation.

SUMMARY

1. The incorporation of labelled glycine, lysine and phosphate into various fractions of rat tissue has been examined at 2 hr. after 1000 r. X-radiation.

2. The uptake of ^{32}P and [2-^{14}C]glycine by rat-thymus DNA was reduced by X-radiation.

3. The incorporation of [2-^{14}C]glycine and [^{14}C]lysine into thymus nucleoproteins was also lowered; uptake into liver nucleoproteins was not affected.

4. Incorporation into cytoplasmic proteins of thymus, liver and pancreas was unaffected or slightly increased.

5. The difference in radiosensitivity between thymus and liver is discussed.

We are grateful to Mr M. J. Corp of the M.R.C. Radiobiological Research Unit, Harwell, for irradiation of the animals and to Dr C. E. Dalgleish and Dr R. W. Dutton for collaboration in the preparation of the ^{14}C-labelled lysine. We also thank Mr J. A. Morris for skilled technical assistance. One of us (J. E. R.) is indebted to The Commonwealth Fund for a Fellowship.

REFERENCES

Allfrey, V. G., Daly, M. M. & Mirsky, A. E. (1955*a*). *J. gen. Physiol.* **38**, 415.

Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1955*b*). *Nature, Lond.*, **176**, 1042.

Allfrey, V. G., Mirsky, A. E. & Stern, H. (1955*c*). *Advanc. Enzymol.* **16**, 411.

Barnes, D. W. H., Esnouf, M. P. & Stocken, L. A. (1956). *Proc. 5th Int. Congr. Radiobiol.* (in the Press).

Bergonie, J. & Tribondeau, L. (1906). *C.R. Acad. Sci., Paris*, **143**, 983.

Brunish, R. & Luck, J. M. (1952). *J. biol. Chem.* **198**, 621.

Butler, J. A. V., Cohn, P. & Crathorn, A. R. (1956). *Proc. 5th Int. Congr. Radiobiol.* (in the Press).

Chargaff, E. (1955). *Nucleic Acids*, vol. 1, p. 307. New York: Academic Press.

Crampton, C. F., Lipshitz, R. & Chargaff, E. (1954). *J. biol. Chem.* **206**, 499.

Daly, M. M., Allfrey, V. G. & Mirsky, A. E. (1955). *J. gen. Physiol.* **39**, 207.

Daly, M. M. & Mirsky, A. E. (1955). *J. gen. Physiol.* **38**, 405.

Deluca, H. A., Rossiter, R. J. & Strickland, K. P. (1953). *Biochem. J.* **55**, 193.

Dounce, A. L. (1955). *Nucleic Acids*, vol. 2, p. 93. New York: Academic Press.

Dounce, A. L., Witter, R. F., Monty, K. J., Pate, S. & Cottone, M. A. (1955). *J. biophys. biochem. Cytol.* **1**, 139.

Drabkin, D. L. (1949). *Arch. Biochem.* **21**, 224.

Dutton, R. W. (1956). *Biochem. J.* **62**, 382.

Frick, G. (1954). *Biochim. biophys. Acta*, **13**, 374.

Garrow, R. & Piper, E. A. (1955). *Biochem. J.* **60**, 527.

Harrington, H. & Lavik, P. S. (1955). *Arch. Biochem. Biophys.* **54**, 6.

Hevesy, G. (1949*a*). *Nature, Lond.*, **163**, 869.

Hevesy, G. (1949*b*). *Nature, Lond.*, **164**, 269.

Hirs, C. H. W., Moore, S. & Stein, W. H. (1952). *J. biol. Chem.* **195**, 669.

Hokin, M. R. (1956). *J. biol. Chem.* **219**, 77.

Hokin, M. R. & Hokin, L. E. (1956). *J. biol. Chem.* **219**, 85.

Kallman, R. F. & Kohn, H. I. (1955). *Radiation Res.* **3**, 77.

Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955). *J. biol. Chem.* **217**, 111.

Miller, L. L., Bly, C. F., Watson, M. L. & Bale, W. F. (1951). *J. exp. Med.* **94**, 431.

Ord, M. G. & Stocken, L. A. (1955). *Rep. 3rd Int. Congr. Biochem., Brussels*, p. 491.

Ord, M. G. & Stocken, L. A. (1956*a*). *Biochem. J.* **63**, 3.

Ord, M. G. & Stocken, L. A. (1956*b*). *Proc. 5th Int. Congr. Radiobiol.* (in the Press).

Richmond, J. E., Altman, K. I. & Salomon, K. (1951). *J. biol. Chem.* **190**, 817.

Schjeide, O. A., Mead, J. F. & Myers, L. S. (1956). *Science*, **123**, 1020.

Sherman, F. & Almeida, A. B. (1956). *Proc. 5th Int. Congr. Radiobiol.* (in the Press).

The Mechanism of Hydrolysis of Adenosine Di- and Tri-phosphate Catalysed by Potato Apyrase

By MILDRED COHN and G. A. MEEK
Department of Biochemistry, University of Oxford

(*Received* 11 *September* 1956)

Under suitable conditions, phosphatases generally catalyse phosphate-transfer reactions (Axelrod, 1956). When these enzymes catalyse the hydrolysis of compounds with an oxygen bridge it has been experimentally demonstrated with ^{18}O that cleavage occurs between P and O, as would be anticipated from the fact that they are phosphoryl-transferring enzymes. By this criterion, adenosine triphosphatases, which have been shown to catalyse the cleavage of the bond between O and the terminal P (Clarke & Koshland, 1953; Cohn, 1956), may be classified as phosphoryl-transferring enzymes.

In the present work, adenosine diphosphate (ADP) and adenosine triphosphate (ATP) have been hydrolysed in $H_2{}^{18}O$ with a potato-apyrase preparation to determine which bond is cleaved and consequently whether the enzyme may act as a phosphoryl-transferring enzyme or as an adenyl- and adenosine diphosphoryl-transferring enzyme. The effect of calcium ions on the reactions has also been investigated.

EXPERIMENTAL

Materials. ATP in the form of the crystalline disodium salt was obtained from Pabst Co. and contained less than 1% of orthophosphate. ADP sodium salt was obtained from Sigma Chemical Co. and contained approximately 10% of orthophosphate. The charcoal (Norit S X 30 Special) used for the adsorption of adenosine phosphates was obtained from Harrington Bros., London. $H_2{}^{18}O$ of 1·7 and 3·1 atom % excess was supplied by the Atomic Energy Research Establishment, Harwell. The potato-apyrase preparation, which was kindly supplied by Dr W. Bartley, had been prepared by the method of Lee & Eiler (1951), and had been stored for several months at −18° (Whittam, Bartley & Weber, 1955). All other chemicals were of AnalaR standard.

Experimental procedures. In the experiments in the rates of hydrolysis, the appropriate substrate was incubated with the enzyme preparation in the presence and absence of Ca^{2+} ions under conditions shown in Fig. 1. Samples of the reaction mixture were removed at various times and the reaction was stopped by the addition of enough 7·5% (w/v) trichloroacetic acid (TCA) to yield a final concentration of 5%. After centrifuging, the supernatant was analysed for liberated orthophosphate.

The hydrolysis of ADP was compared under the same conditions (1) with no additions, (2) in 0·0025 M-ethylenediaminetetra-acetate (EDTA) and (3) with 0·005 M-$CaCl_2$. The rates were approximately the same with EDTA and no additions and considerably higher with $CaCl_2$.

In the series of hydrolysis experiments in $H_2{}^{18}O$, the reaction components were added in a manner designed to minimize dilution of ^{18}O. Each tube contained 0·2 ml. of M-sodium succinate buffer made up in $H_2{}^{18}O$; all other components were added as solids, except 25 µl. of KOH solution used for neutralization and the amounts of enzyme preparation indicated in Table 1. Enough $H_2{}^{18}O$ was added in each tube to bring the final volume to 4 ml. Each hydrolysis was allowed to proceed for a sufficient time to yield enough orthophosphate for ^{18}O analysis but not to exceed

Table 1. *Hydrolysis of ATP and ADP in* $H_2{}^{18}O$

Reaction mixtures nos. 1–4 had a total volume of 4 ml.; no. 5, 2 ml. All contained sodium succinate buffer, pH 6·5 (0·05 M), and enzyme preparation, 0·1 ml./ml. of reaction mixture except no. 2, which had 0·05 ml./ml. of reaction mixture. Temp., 40°.

				^{18}O concn. (atom % excess)		
				H_2O recovered from reaction mixture	Inorganic phosphate	
No.	Substrate	Time (min.)	Hydrolysis of terminal P (%)		Found	Calc. $O/P_{terminal}$*
1	ATP (0·017 M)	40	70·5	1·13	0·305	0·283
2	ATP (0·016 M) + $CaCl_2$ (~0·013 M)	10	62·0	1·17	0·315	0·293
3	ADP (0·011 M)	240	73	2·63	0·667	0·665
4	ADP (0·011 M) + $CaCl_2$ (~0·012 M)	180	>90	2·60	0·669	0·650
5	KH_2PO_4	240	—	2·53	0·0	—

* The calculated value is equal to one-quarter of the atom % excess in the water of the reaction mixture.

1 mole of orthophosphate/mole of substrate. The duration of each hydrolysis and the amount of orthophosphate liberated is listed in Table 1. At the end of the experiment, 0·1 ml. samples of each reaction mixture were withdrawn and reserved for ^{18}O analysis of the water in the medium.

In the hydrolysis of ATP (Exps. 1 and 2, Table 1), the reaction was stopped by the addition of 1·33 ml. of 20% (w/v) TCA. After centrifuging, the supernatants were analysed for orthophosphate and 10 min.-hydrolysable phosphate to determine the amount of hydrolysis. Charcoal (1·2 g.) which had been acid-washed (Lipkin, Talbert & Cohn, 1954) and tested for its capacity to adsorb nucleotides, was added to each supernatant to remove the nucleotides. The inorganic orthophosphate was precipitated as the barium salt and subsequently isolated as the monopotassium salt for ^{18}O analysis as described by Cohn (1953).

In the experiment with higher concentrations of $H_2{}^{18}O$ (nos. 3, 4 and 5, Table 1), the reaction was stopped by heating in a stoppered tube in a boiling-water bath for 2 min. The enriched water was recovered by vacuum distillation at room temperature. The residue was suspended in 2 ml. of 5% (w/v) TCA and centrifuged. The precipitate was washed with 1 ml. of 5% (w/v) TCA and the washing was added to the supernatant. The inorganic orthophosphate was precipitated as the magnesium ammonium salt and subsequently as the monopotassium salt (Cohn, 1953).

Analytical methods. Inorganic orthophosphate was analysed by the Fiske–Subbarow method (1925). The water and orthophosphate were analysed in the form of CO_2 for ^{18}O as described by Cohn (1953). The isotope ratios were determined on a mass spectrometer of the type described by Graham, Harkness & Thode (1947).

RESULTS

Effect of calcium ions on the rate of hydrolysis

As shown in Fig. 1, under the conditions specified, the rate of ATP hydrolysis is increased six- to seven-fold by $CaCl_2$ and that of ADP approximately threefold. Other investigators, e.g. Krishnan (1949), have found similar stimulatory effects of calcium. Nguyen-Van Thoai, Roche & Tran-Thi (1954) prepared a purified form of the enzyme and found no effect of Ca^{2+}, Mg^{2+} or Mn^{2+} ions over a concentration range of 10^{-2} to 10^{-4} M. One interpretation of the discrepancy is that the potato-apyrase preparation of Lee & Eiler (1951) used in the present investigation might be a mixture of enzymes, including one stimulated by Ca^{2+} ions, which has been removed in the purification procedure of Nguyen-Van Thoai *et al.* (1954). However, both preparations exhibit the same relationship to substrates, namely that no ADP is hydrolysed until all the ATP has been converted into ADP (cf. Whittam *et al.* 1955). The negative effect of bivalent ions found by Nguyen-Van Thoai *et al.* (1954) with the purified enzyme may be misleading in view of the fact that the assay system contained approximately 0·002 M-ATP and 0·1 M-histidine buffer; the latter may be responsible for binding all the calcium in the reaction mixture. The enzyme preparation was assayed for its hydrolytic activity with adenylic acid (AMP) as substrate. With 0·1 ml. of enzyme preparation/ml. of reaction mixture and in the presence of 0·005 M-$CaCl_2$ and 0·025 M-AMP, 7 and 30% hydrolysis occurred in 1 and 4 hr. respectively.

Hydrolysis in $H_2{}^{18}O$

From the ^{18}O content of the orthophosphates liberated in the hydrolyses as shown in Table 1, it is apparent that one oxygen of the four was incorporated from the medium. The cleavage of ATP and ADP with or without Ca^{2+} ions always occurs between O and the terminal P. Whether the potato-apyrase preparation is a single enzyme or a mixture of enzymes, the cleavage of the same bond is catalysed. The orthophosphate formed in experiments 3 and 4, which proceeded for 4 and 3 hr. respectively, was probably in part liberated from adenylic acid; the experiments thus indicate that the phosphatase contaminating the preparation catalyses the cleavage of the bond between O and P in adenylic acid. The control with KH_2PO_4 (Expt. 5) indicates that the preparation does not catalyse any significant exchange of orthophosphate and water.

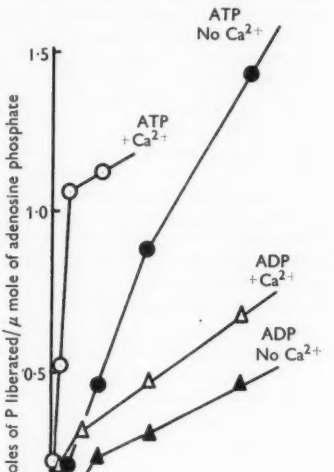


Fig. 1. Rates of hydrolysis of ADP and ATP with and without $CaCl_2$. The reaction mixtures contained, in a total volume of 2 ml., 0·1 ml. of enzyme preparation and final concentrations of sodium succinate buffer, pH 6·5, 0·05 M; ATP (0·008 M) or ADP (0·006 M); $CaCl_2$ (0·01 M). Temp., 40°. Orthophosphate values at 2 hr. are somewhat high owing to hydrolysis of AMP.

DISCUSSION

Many hydrolytic enzymes, including the phosphatases, catalyse group-transfer reactions so that hydrolysis may be considered a particular case of the general reaction when water is the acceptor. A number of non-hydrolytic enzymes has been described which catalyse adenyl transfer rather than phosphoryl transfer (1) from ADP, polynucleotide phosphorylase (Grunberg-Manago & Ochoa, 1955); (2) from ATP, acetate activation (Berg, 1955), pantoate activation (Maas & Novelli, 1953), amino acid activation (Hoagland, 1954) and others. The most direct method for ascertaining which group is being transferred in a hydrolytic reaction is the determination of the site of bond cleavage by hydrolysis in $H_2{}^{18}O$ (for review, see Axelrod, 1956). In the hydrolysis of the terminal phosphate of ATP or ADP, a P–O bond is broken when cleavage occurs on either side of the oxygen. However, the two alternative types of enzymic catalysis are basically different, in mechanism and in function; in one a phosphoryl group is transferred and in the other an adenosine diphosphoryl or adenyl group is transferred. The enzymes from muscle catalysing the hyrolysis of ATP have been shown to effect the cleavage of the bond between the terminal P and O (Clarke & Koshland, 1953; Cohn, 1956); ATP may be considered as a donor of phosphoryl groups in these reactions.

ATP may be either a donor of phosphoryl groups, as in kinase reactions, or a donor of adenyl groups, as in the acetate-activation reaction. In the former group of reactions, the bond between the terminal P and O is ruptured (Harrison, Boyer & Falcone, 1955; Cohn, 1956) and in the latter, the bond between the P of the adenyl moiety and O is cleaved (Boyer, Koeppe & Luchsinger, 1956). There are no known reactions in which ATP acts as a donor of adenosine diphosphoryl groups.

There are few reactions in which ADP acts as a donor. In the adenylate kinase reaction, ADP is a donor of phorphoryl groups as indicated by ^{18}O experiments (Cohn, 1956). In the polynucleotide phosphorylase reaction (Grunberg-Manago & Ochoa, 1955), ADP appears to act as a donor of adenyl groups, although the detailed mechanism of this reaction has not yet been elucidated.

From the present study it is clear that the potato-apyrase preparation which catalyses the hydrolysis of ATP and ADP can catalyse solely the transfer of phosphoryl groups from these substrates. Furthermore the catalysis by Ca^{2+} ions does not change the mechanism of the reaction with regard to the site of bond cleavage.

SUMMARY

1. The hydrolysis of ATP and ADP catalysed by a potato-apyrase preparation has been investigated in $H_2{}^{18}O$. It was found that the cleavage occurs between O and the terminal P of either substrate both in the presence and absence of calcium ions.

2. The implications of these findings for the mechanisms of these reactions is discussed.

This work was carried out during the tenure by one of us (M.C.) of an Established Investigatorship of the American Heart Association.

REFERENCES

Axelrod, B. (1956). *Advanc. Enzymol.* **17**, 159.
Berg, P. (1955). *J. Amer. chem. Soc.* **77**, 3163.
Boyer, P. D., Koeppe, O. J. & Luchsinger, W. W. (1956). *J. Amer. chem. Soc.* **78**, 356.
Clarke, E. & Koshland, D. E. jun. (1953). *Nature, Lond.*, **171**, 1223.
Cohn, M. (1953). *J. biol. Chem.* **201**, 735.
Cohn, M. (1956). *Biochim. biophys. Acta*, **12**, 172.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Graham, R. L., Harkness, A. L. & Thode, H. G. (1947). *J. sci. Instrum.* **24**, 119.
Grunberg-Manago, M. & Ochoa, S. (1955). *J. Amer. chem. Soc.* **77**, 3165.
Harrison, W. H., Boyer, P. D. & Falcone, A. B. (1955). *J. biol. Chem.* **215**, 303.
Hoagland, M. B. (1954). *Biochim. biophys. Acta*, **16**, 288.
Krishnan, P. S. (1949). *Arch. Biochem.* **20**, 272.
Lee, K. H. & Eiler, J. J. (1951). *Science*, **114**, 393.
Lipkin, D., Talbert, P. T. & Cohn, M. (1954). *J. Amer. chem. Soc.* **76**, 2871.
Maas, W. K. & Novelli, G. D. (1953). *Arch. Biochem. Biophys.* **43**, 236.
Nguyen-Van Thoai, Roche, J. & Tran-Thi, An. (1954). *Bull. Soc. chim. Biol., Paris*, **36**, 529.
Whittam, R., Bartley, W. & Weber, G. (1955). *Biochem. J.* **59**, 590.

Inositol in Seminal Plasma

By E. F. HARTREE
A.R.C. Unit of Reproductive Physiology and Biochemistry, Molteno Institute, University of Cambridge

(*Received 7 November* 1956)

Inositol (*meso*inositol or *myo*inositol) occurs very widely in plants, animals and micro-organisms (Fleury & Balatre, 1947; Courtois, 1951; Hawthorne & Chargaff, 1954; Mann, 1954*a*). It occurs in the free state as well as in the form of inositol phosphatides, while in plants it is often present as the hexaphosphate, phytic acid. Treatment of liver and nervous tissue by the standard procedure of Schmidt & Thannhauser (1945) gives ribonucleic acid (RNA) fractions contaminated by materials which yield inositol phosphates on hydrolysis. According to Hutchison *et al.* (1956) these consist of peptide-inositol phosphates derived from protein-bound inositol phosphates. On hydrolysis of such complexes inositol is usually liberated as the monophosphate, but Folch's (1952) analysis of neurokeratin from ox-brain showed that the inositol was present mainly as a diphosphate forming part of a lipoprotein. Of apparently more restricted distribution are free inositol monophosphate which has been isolated from guinea-pig liver (Hawthorne & Hübscher, 1956) and an inositol galactoside which has been obtained from sugar-beet molasses (Brown & Serro, 1953).

The inositol contents of the majority of plant and animal tissues, estimated after hydrolysis, vary between 10 and 100 mg./100 g. fresh weight (Mann, 1954*a*). An exceptionally rich source is the seminal vesicle secretion of the boar, in which Mann (1951, 1954*a*) found inositol levels of about 2400 mg./100 ml. In this case the inositol is present largely, if not entirely, in the free state. The present survey of the inositol content of semen and of secretions from the accessory organs of the male reproductive tract was undertaken as an introduction to the wider problems of the function and metabolism of inositol in the male reproductive organs.

MATERIALS

Semen of boar, bull, rabbit, ram and stallion was collected by means of the artificial vagina (Walton, 1945), and was supplied by the Animal Research Station, Huntingdon Road, Cambridge. Human semen from donors at the London Fertility Clinic was made available through the kidness of Dr H. Davidson. Except for the samples that are specified below, the semen was centrifuged within 2 hr. of collection in an angle head at 15 000 *g* for 20 min. The resulting clear plasma was preserved at −15° if not immediately analysed. With ram semen the packed spermatozoa in the centrifuge tube were washed with Ca-free Ringer solution (Mann, 1946). Some semen samples (nos. 16–19, 23, 51, 52) were stored at −15° as whole semen. After thawing, the plasma was obtained as above.

The materials listed in Table 3 were obtained immediately after the death of the animals. Epididymal semen, seminal vesicle secretion and ampullar secretion were expressed from the organs, the seminal plasma being separated as before. The hedgehog prostates were ground with an equal weight of water, deproteinized with 5 vol. of ethanol and the clear fluid concentrated so that 1 ml. was equivalent to 1 g. of tissue.

METHODS

Microbiological assay of inositol. The technique adopted was that of Campling & Nixon (1954), which utilizes the yeast *Kloeckera brevis* (National Collection of Yeast Cultures no. 328). The samples to be analysed were added to 2·5 ml. of inositol-free medium in screw-stoppered bottles, diluted to 5 ml. with water, sterilized for 20 min. at 120° and inoculated with one drop of a very dilute *K. brevis* suspension. Duplicate sets of standards (0–5 μg. of inositol) were set up on each occasion to provide data for a standard curve relating turbidity to inositol content. After 65–70 hr. incubation at 25° the growth was estimated by a Hilger absorptiometer with red filters (transmission peak: 675 mμ) or with an EEL nephelometer. No growth occurred when the concentration of inositol was less than 0·1 μg./5 ml. (Fig. 1). For estimations within the range

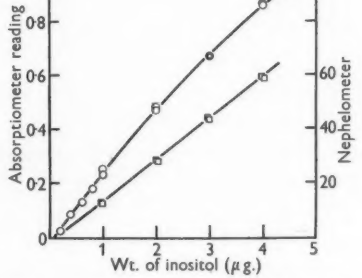


Fig. 1. Relationship between growth of *K. brevis* and concentration of inositol. Turbidity measured in the Spekker absorptiometer with red filters (○) or in the EEL nephelometer (□).

0–1 μg. of inositol the sensitivity of the method was increased by measuring growth in terms of the extinction at 800 mμ of the yeast suspension in 2 cm. spectrophotometer cells. If red light is used the slight brown colour of some hydrolysed samples does not interfere. The standard deviation did not exceed 4%.

Free inositol. The most reliable method for deproteinization of seminal plasma and of the various secretions that were investigated proved to be that developed by Somogyi (1945) for blood. To 0·5 ml. of seminal plasma was added 7·5 ml. of water and 1·0 ml. of 0·15M-$Ba(OH)_2$. After mixing, the solution was treated with 1·0 ml. of exactly equivalent $ZnSO_4$ solution. (Equivalence is determined by titrating the zinc solution, considerably diluted with water, with the baryta in the presence of phenolphthalein.) After vigorous shaking the mixture was frozen at −15° overnight, thawed and centrifuged. The freezing and thawing are not part of Somogyi's method and is unnecessary for deproteinizing blood. In the present work, however, omission of this step sometimes resulted in slightly opalescent solutions.

Total inositol. Samples were hydrolysed by refluxing for 16 hr. in HCl (reagent grade). The final concentration of acid was 6N. As a rule 0·2 ml. of seminal plasma (or other secretion), alternatively 10–20 mg. of dry material, was diluted to a final volume of 5·5 ml. and boiled in test tubes fitted with ground-in air condensers. In some cases, especially boar seminal plasma, the solution becomes very dark. Furthermore, sufficient inorganic phosphate may be released to give rise to a precipitate of heavy-metal phosphates when the hydrolysate is added to the culture medium. The following procedure reduced the colour and the phosphate concentration to a negligible level.

The hydrolysate was evaporated to dryness on a steam bath, treated with 5 ml. of water and 50 mg. of talc (British Drug Houses Ltd.) previously washed with hot 6N-HCl, and evaporated again to dryness. The cooled residue was stirred with 5 ml. of $Ca(HCO_3)_2$ solution. This was prepared by saturating an aqueous suspension of $CaCO_3$ with CO_2 for 1 hr. at 0° and allowing excess of $CaCO_3$ to settle. The solution was stored at 5°. The bicarbonate-treated residue was slowly heated to 100° and then evaporated to dryness. The residue was suspended in a known volume (usually 10 ml.) of water and centrifuged.

Known amounts of inositol added to plasma before deproteinization were recovered completely. Similar experiments using the procedure for total inositol indicated a recovery of at least 96%.

RESULTS

Specificity of meso*inositol in the microbiological assay with* Kloeckera brevis

Woolley (1941*a*) found that with *Saccharomyces cerevisiae* the microbiological method was highly specific for *meso*inositol, the isomeric cyclitols and their ethers being virtually inactive. The inositol-requiring mutants of *Neurospora crassa* can also be used for the assay (Beadle, 1944).

The use of *K. brevis* for the estimation of inositol was proposed by Burkholder, McVeigh & Mayer (1944) and the technique has been developed by Northam & Norris (1952). Although 3 days' incubation are required for maximal growth of this yeast instead of 16 hr. with *Saccharomyces* spp. it has two important advantages over the latter. *K. brevis* will not proliferate in absence of inositol and its growth is not inhibited by choline (Lovern & Olley, 1953).

The high specificity of *meso*inositol shown by *K. brevis* is illustrated in Table 1. The results were obtained by comparing the growth response to 1, 0·2 and 0·04 mg. of the substances listed with the growth response to 2 μg. of *meso*inositol.

Conditions for complete hydrolysis of bound inositol by acid hydrolysis

Preliminary experiments were carried out with ram seminal plasma in which it was found that only a small proportion of the inositol was present in the free state. While assays of free inositol yielded concordant results, estimations of total inositol were

Table 1. *Specificity of* meso*inositol as growth factor for* Kloeckera brevis *in a medium containing glucose and asparagine as carbon sources*

		Relative activity
a	*meso*Inositol	100
	*epi*Inositol	3
	(−)-Inositol	0·2
	Scyllo*meso*inososse	4
	Quebrachitol	0·5
	(−)-Virburnitol	4
	Inositol monophosphate: probably the 5-isomer (Hawthorne, 1955)	9

b Inactive (<0·04% of activity of *meso*inositol)

Arabinose	Dulcitol
Cellobiose	Erythrol
Fructose	Mannitol
Fucose	Pentaerythritol
Galactose	(+)-Quercitol
Lactose	Sorbitol
Maltose	Scyllitol
Mannose	
Xylose	

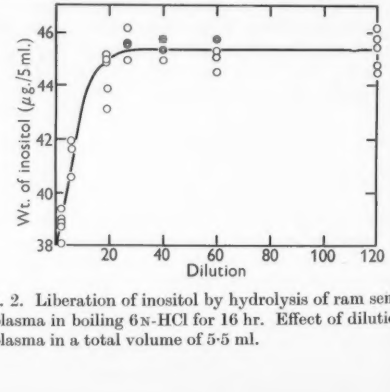


Fig. 2. Liberation of inositol by hydrolysis of ram seminal plasma in boiling 6N-HCl for 16 hr. Effect of dilution of plasma in a total volume of 5·5 ml.

not reproducible. This lack of reproducibility was traced to variations in the dilution of seminal plasma in the hydrolysis mixture, an effect that does not appear to have been recorded previously. Thus 16 hr. boiling of 5·5 ml. of 6N-HCl containing 1 ml. of plasma yielded proportionately less free inositol than did a parallel experiment with 0·2 ml. of plasma. It was found that maximal liberation of inositol from both ram and boar seminal plasma took place only when the dilution of seminal plasma was greater than 25 (Fig. 2).

The liberation of inositol from lipids of brain (Folch & Woolley, 1942) and haddock flesh (Lovern & Olley, 1953) is reported to be complete after 6 hr. boiling with 6N-HCl. Since no information is available on the nature of the bound inositol in the materials now being reported on, the effect of time of hydrolysis was determined. In the case of ram and boar seminal plasma complete hydrolysis requires 16 hr. (at a dilution of 25) although 95–97% of the bound inositol is liberated during the first 6 hr.

The inositol level, after hydrolysis, in a mixture of seminal plasma and inositol was, in several experiments, equal to the sum of the separately determined inositol in the two components. A similar agreement in levels of free inositol was observed if the standard deproteinization procedure was substituted for hydrolysis. Thus seminal plasma is devoid of any inhibitors of the growth of *K. brevis*.

Reaction of inositol phosphatides to the standard procedures for hydrolysis and deproteinization

On the assumption that the bound inositol in the samples under examination was largely lipid inositol, tests were carried out to determine whether inositol lipids would be co-precipitated with proteins during deproteinization with the Ba–Zn reagent. A sample of mixed serine and inositol phosphatides, obtained from ox-liver, was supplied by Dr J. N. Hawthorne.

Complete hydrolysis of 1 mg. of mixed phosphatides under the standard conditions required 16 hr., the yield of free inositol being 110 μg. The assay of 1 mg. of untreated phosphatide elicited a growth equivalent to 2·8 μg. of inositol. Thus the inositol equivalent of inositol phosphatide is only 2·5% of that of the hydrolysed phosphatide. It is very unlikely that the phosphatide contains free inositol, but slight hydrolysis probably occurred during sterilization at 120°. If the mixed phosphatides were subjected to the deproteinization procedure with $Ba(OH)_2$ and $ZnSO_4$ and the clear solution was subsequently hydrolysed, free inositol could be detected. In such experiments 10 mg. of phosphatide (containing 1100 μg. of bound inositol) yielded 184 μg. of free inositol. Thus 17% of the inositol phosphatide was not precipitated by the barium–zinc reagents. However, such incomplete precipitation would introduce a negligible error in estimation of free inositol, since the inositol equivalent of unhydrolysed inositol phosphatide is only 2·5%. In fact, the addition of inositol phosphatide to seminal plasma does not affect the level of free inositol determined after deproteinization. Thus 0·5 ml. of stallion seminal plasma (Table 2, sample 13), and 0·5 ml. of the same plasma in which 2 mg. of mixed phosphatides had been dissolved, were each deproteinized and samples of the clear solutions subsequently hydrolysed. Before hydrolysis the free inositol assays were 198 and 200 μg. respectively, while after hydrolysis the inositol contents were 197 and 235 μg. respectively. The production of 35 μg. excess of inositol on hydrolysis of the deproteinized mixture of plasma and 2 mg. of mixed phosphatides again indicates that about 17% of the inositol phosphatide was not precipitated during deproteinization. However, the effect of the remaining phosphatide in the estimation of free inositol was negligible.

It was suggested (Mann, 1954*a*) that seminal vesicle inositol may derive from a phosphorylated precursor and more recently free inositol monophosphate has been detected in liver (Hawthorne & Hübscher, 1956). However, Hutchison *et al.* (1956) found that bull and sea-urchin sperm was unique, among a range of cells and tissues, in yielding 'RNA fractions' devoid of inositol phosphates. The barium salts of phosphorylated inositols containing more than one phosphate group are insoluble in water while those of the monophosphates require alcohol for their precipitation (Posternak & Posternak, 1929). Thus inositol monophosphates might conceivably be present in the protein-free filtrates after deproteinization. A sample of the monophosphate obtained from phytic acid was made availabie by Dr J. N. Hawthorne. This substance is probably the 5-phosphate (Hawthorne & Chargaff, 1954; Hawthorne, 1955). The assay of inositol monophosphate (containing 1 mg. of bound inositol) gave an inositol equivalent of 90 μg. When the monophosphate was subjected to deproteinization the equivalent fell to 7·5 μg., while deproteinization followed by hydrolysis gave an equivalent of 60 μg. Thus although about 6% of inositol monophosphate remains in solution after barium–zinc treatment its effect upon the assay of free inositol after deproteinization would be negligible, i.e. less than 1% of the effect detectable after hydrolysis.

Distribution of inositol

Inositol has been detected in every sample of seminal plasma which has so far been examined (Table 2). While the total inositol content of boar seminal plasma normally exceeds 600 mg./100 ml., that of bull, human, ram and stallion plasma is less

than 100 mg./100 ml. Within a species the inositol level in the plasma varies considerably but the ratio of free to combined inositol appears, on the basis of the present limited series of estimations, to be approximately constant for each species. Thus in boar and human seminal plasma almost the entire inositol is present in the free state. The proportions of inositol in the free state in the plasma of bull and stallion semen are 55 and 85% respectively while for ram plasma, where the variations are greater, the figure is of the order of 30%. When two ejaculates were collected from a bull in a single day, the inositol concentration was markedly lower in the second ejaculate.

While the seminal vesicles are the main source of free inositol in boar semen (Mann, 1951, 1954*a*), the epididymal seminal plasma contributes significant quantities of both free and combined inositol; the inositol content of boar epididymal plasma is of the same order as the inositol content of seminal plasma in other species. Monkey seminal vesicle secretion is also comparatively rich in free inositol. On the other hand, the seminal vesicle secretion of the bull appears to contain rather less of both free and combined inositol than does the seminal plasma. Among the secretions so far examined the richest in combined inositol was a sample of stallion ampullar secretion (Table 3).

Table 2. *Free and total inositol in seminal plasma*

The two pairs 16, 17 and 18, 19 each represent first and second ejaculates respectively collected from the one animal on the same day. Sample 23 was from a bull which always provides semen containing separate sperm heads and sperm tails.

Species	Sample no.	Animal no.	Inositol (mg./100 ml.) Free	Total
Boar	2	I	382	—
	5	I	625	605
	7	II	607	725
Bull	12	I	27·2	48·0
	16	II	45·9	83·8
	17	II	28·1	44·9
	18	III	45·0	88·5
	19	III	38·6	61·4
	23	IV	24·6	52·1
Human	51	*	50·0	53·6
	52	*	57·3	62·8
Rabbit	34	I	21·0	27·7
Ram	1	*	9·7	37·3
	4	*	16·1	—
	6	*	14·6	39·0
	8	*	—	45·4
	9	*	7·4	41·5
Stallion	3	I	24·4	—
	10	I†	27·5	33·6
	11	I	19·0	24·0
	13	I	39·6	43·5
	14	I	47·3	51·2
	15	I	29·5	35·0

* Ten or more pooled ejaculates. † Three pooled ejaculates from the one animal.

Table 3. *Free and total inositol in male accessory secretions*

Species	Secretion or tissue	Sample no.	Inositol (mg./100 ml.) Free	Total
Boar	Epididymal seminal plasma	20	36·5	52·2
Bull	Seminal vesicle secretion	21	40·0	54·9
		22	18·7	25·5
Guinea pig	Seminal vesicle secretion	44	19·2	26·1
Monkey	Seminal vesicle secretion	26	145	141
Hedgehog	Prostate I and II*	32	17·5†	—
	Prostate III*	33	1·5†	—
Stallion	Ampullar secretion	25	20·4	93

* Whole gland. † mg./100 g. fresh weight.

The total inositol content of ram spermatozoa was found to be 180 mg./100 g. dry weight. Nevertheless, the lipids isolated from the same source by Lovern, Olley, Hartree & Mann (in preparation) were reported to be free from inositol lipids, although the analytical procedure used by these authors would have detected a quantity of inositol equivalent to as little as 10 mg./100 g. dry weight of spermatozoa. The initial stage in the separation of the lipids, exhaustive extraction with chloroform–methanol, removed less than half the total inositol in the dried spermatozoa. Of the inositol present in the resulting crude lipids virtually all was removed when they were subjected to the purification procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951) followed by the cellulose-column treatment of Lea & Rhodes (1953) (unpublished results). Later experiments have shown that the residue remaining after chloroform–methanol extraction contains considerable quantities of bound inositol.

DISCUSSION

Apart from Mann's (1951, 1954*a*) published figures for the inositol content of vesicular secretion and semen of the boar the only previous information on inositol in semen was that human semen contains less than 100 mg./100 ml. (Nixon, 1952). The present results show that seminal inositol is of widespread occurrence. Although in most animals the level in the seminal plasma is 10–40 times lower than in boar seminal plasma, such levels are considerably higher than those characteristic of other body fluids, e.g. blood and cerebrospinal fluid (Table 4). Thus inositol at this higher concentration can be classified with citric acid (Scherstén, 1936) and fructose (Mann, 1946) as a characteristic component of semen.

On the basis of configurational relationships Fischer (1945) put forward the suggestion that inositol may be an intermediate in the interconversion of glucose and fructose. Since inositol is not readily degraded in animal tissues it could, according to this hypothesis, function as a non-metabolizable carbohydrate reserve. In this connexion can be cited certain parallelisms in the distribution of inositol and fructose: thus foetal blood and semen each contain unusually high concentrations of both substances (Table 4).

Inositol is well known as a growth factor for certain micro-organisms, while Woolley (1940, 1941*b*) has shown that it is an essential dietary factor for rats; the main deficiency symptom being alopecia. However, in later experiments Woolley (1942) found that the response of the rats to inositol-deficient diets was very irregular, a result which was traced to the ability of the intestinal flora to synthesize inositol. Availability of inositol from this source would explain why Needham (1924) failed to lower the inositol content of tissues of rats on an inositol-deficient diet. On the other hand, Eagle, Oyama, Levy & Freeman (1956) have demonstrated that inositol is a growth factor for certain normal and malignant cells in tissue culture. Of 19 cell lines (17 human, 2 mouse) 16 were unable to grow in absence of inositol and required concentrations of 10^{-5} to 10^{-6} M for optimum growth. Another established function for inositol is that of a lipotropic factor. Gavin & McHenry (1941) showed that inositol can prevent the formation of fatty livers in rats on high-cholesterol diets.

It is not possible at present to ascribe any metabolic function to inositol in semen or, in fact, in any other animal tissue. Feeding inositol to rats gives rise to no increase in liver glycogen but at the same time very little of the inositol is excreted in the urine. Posternak, Schopfer & Reymond (1955) have demonstrated the conversion by rats of deuterium-labelled inositol into 6-C labelled glucose, and Halliday & Anderson (1955) similarly established the formation of labelled inositol after feeding [1-^{14}C]glucose. However the rates of conversion were very low in both cases.

The materials used in the present work were, when not obtained from living animals, dissected out immediately after death. The importance of rapid

Table 4. *Mean values for fructose and free inositol in semen, cerebrospinal fluid, and maternal and foetal blood*

Apart from the results for the free inositol content of human and ram seminal plasma the results are quoted from the following papers: Bacon & Bell (1948); Campling & Nixon (1954); Cole & Hitchcock (1946); Eastham & Keay (1952); Mann (1954*b*); Nixon (1955); Wallenfels (1951).

	Fructose (mg./100 ml.)	Free inositol (mg./100 ml.)
Ram seminal plasma	300	15
Sheep blood, maternal	1	1·6
Sheep blood, foetal	100 (mid-pregnancy) 60 (at term)	20
Human seminal plasma	300	50
Human blood	2	0·6
Human cerebrospinal fluid	3·8	2·7

removal is emphasized by the work of Nixon (1955) who found dramatic rises in the inositol content of human cerebrospinal fluid within 12 hr. of death. These increases are ascribed to the hydrolysis of phospholipids present in the cytoplasm of the central nervous system. *Post mortem* autolytic rises of free inositol in muscle have also been reported (see Nixon, 1955).

The role of fructose as a nutrient for spermatozoa has been fully established (Mann & Lutwak-Mann, 1948), but attempts to demonstrate a similar role for inositol have been unsuccessful. Although fructose induces motility in epididymal spermatozoa, inositol does not. Furthermore, addition of inositol to boar epididymal semen or to washed sperm suspensions in Ringer–fructose solution does not bring about any change in respiration or fructolysis (Mann, 1954*a*).

The concentration of free inositol in boar vesicular secretion shows very little variation. Analysis of the fluids obtained from the vesicles of five boars gave an average figure of 2414 mg./100 ml. with a standard deviation of only 8 % (Mann, 1954*a*). On the other hand, the total volume of fluid obtained from a boar, as well as the combined weight of pairs of vesicles, varied more than tenfold. Since almost all the inositol in boar seminal plasma derives from the seminal vesicles, the inositol level in the plasma may prove to be a useful index of the contribution of the vesicular secretion to the ejaculated semen.

It has been pointed out by Mann (1954*a*) that boar vesicular secretion, unlike the other body fluids, is almost devoid of sodium chloride, and he suggests that the function of inositol here, and to a lesser degree in the seminal plasma, is to maintain the osmotic pressure. However, it is now established that although inositol is widely distributed in the accessory secretions which make up the seminal plasma, it is present at concentrations much lower than those characteristic of the boar (compare Table 2 of this paper with Table 4 of Mann, 1954*b*). It must therefore be assumed that, while the osmotic role of inositol in boar semen is important, this substance may well have some more specific function in semen.

SUMMARY

1. Free and total inositol have been assayed in seminal plasma, and in some accessory organs of the male reproductive tract, by a microbiological method which utilizes the yeast *Kloeckera brevis*.

2. Boar seminal plasma contains 600–700 mg. of inositol/100 ml., most of which is in the free state. Bull, human, rabbit, ram and stallion seminal plasma contain less than 100 mg./ml. In all species the inositol concentration is much greater than in blood and cerebrospinal fluid.

3. It is suggested that the inositol content of boar semen can be taken as an index of the secretory activity of the seminal vesicles.

4. A parallelism between the levels of fructose and inositol in body fluids supports the view that inositol may function as an intermediate in the interconversion of glucose and fructose, but at present there is no evidence for any metabolic role of inositol in semen.

I wish to express my thanks to Dr J. N. Hawthorne for gifts of inositol monophosphate and inositol phosphatide, and to Professor Th. Posternak for his gift of the homologous compounds listed in Table 1. I also wish to thank Dr T. Mann, F.R.S., for much helpful advice during the course of this investigation.

REFERENCES

Bacon, J. S. D. & Bell, D. J. (1948). *Biochem. J.* **42**, 397.
Beadle, G. W. (1944). *J. biol. Chem.* **156**, 683.
Brown, R. J. & Serro, R. F. (1953). *J. Amer. chem. Soc.* **75**, 1040.
Burkholder, P. R., McVeigh, I. & Mayer, D. (1944). *J. Bact.* **48**, 385.
Campling, J. D. & Nixon, D. A. (1954). *J. Physiol.* **126**, 71.
Cole, S. W. & Hitchcock, M. W. S. (1946). *Biochem. J.* **40**, li.
Courtois, J. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1075.
Eagle, H., Oyama, V. I., Levy, M. & Freeman, A. (1956). *Science*, **123**, 845.
Eastham, M. D. & Keay, K. R. (1952). *Biochem. J.* **50**, iv.
Fischer, H. O. L. (1945). *Harvey Lect.* series XL, p. 156.
Fleury, P. & Balatre, P. (1947). *Les Inositoles, Chimie et Biochimie.* Paris: Masson.
Folch, J. (1952). *Phosphorus Metabolism*, vol. 2, p. 186. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Folch, J., Ascoli, I., Lees, M., Meath, J. A. & LeBaron, F. N. (1951). *J. biol. Chem.* **191**, 833.
Folch, J. & Woolley, D. W. (1942). *J. biol. Chem.* **142**, 963.
Gavin, G. & McHenry, E. W. (1941). *J. biol. Chem.* **139**, 485.
Halliday, J. W. & Anderson, L. (1955). *J. biol. Chem.* **217**, 797.
Hawthorne, J. N. (1955). *Biochim. biophys. Acta*, **18**, 389.
Hawthorne, J. N. & Chargaff, E. (1954). *J. biol. Chem.* **206**, 27.
Hawthorne, J. N. & Hübscher, G. (1956). *Biochem. J.* **64**, 53P.
Hutchison, W. C., Crosbie, G. W., Mendes, C. B., McIndoe, W. M., Childs, M. & Davidson, J. N. (1956). *Biochim. biophys. Acta*, **21**, 44.
Lea, C. H. & Rhodes, D. N. (1953). *Biochem. J.* **54**, 467.
Lovern, J. A. & Olley, J. (1953). *Biochem. J.* **55**, 686.
Mann, T. (1946). *Biochem. J.* **40**, 481.
Mann, T. (1951). *Nature, Lond.*, **168**, 1043.
Mann, T. (1954*a*). *Proc. Roy. Soc.* B, **142**, 21.
Mann, T. (1954*b*). *The Biochemistry of Semen.* London: Methuen.

Mann, T. & Lutwak-Mann, C. (1948). *Biochem. J.* **43**, 266.
Needham, J. (1924). *Biochem. J.* **18**, 891.
Nixon, D. A. (1952). M.Sc. Thesis: London University.
Nixon, D. A. (1955). *J. Physiol.* **129**, 272.
Northam, B. E. & Norris, F. W. (1952). *J. gen. Microbiol.* **7**, 245.
Posternak, S. & Posternak, T. (1929). *Helv. chim. acta*, **12**, 1165.
Posternak, T., Schopfer, W. H. & Reymond, D. (1955). *Helv. chim. acta*, **38**, 1283.
Scherstén, B. (1936). *Skand. Arch. Physiol.* **74**, suppl. 7.
Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Wallenfels, K. (1951). *Naturwissenschaften*, **38**, 238.
Walton, A. (1945). *Notes on Artificial Insemination of Sheep, Cattle and Horses.* London: Holborn Surgical Instrument Co.
Woolley, D. W. (1940). *J. biol. Chem.* **136**, 113.
Woolley, D. W. (1941*a*). *J. biol. Chem.* **140**, 461.
Woolley, D. W. (1941*b*). *J. biol. Chem.* **139**, 29.
Woolley, D. W. (1942). *J. exp. Med.* **75**, 277.

Tissue Components of the Domestic Fowl

1. THE D-GLUCOSE CONTENT OF WHOLE BLOOD

BY D. J. BELL
Agricultural Research Council Poultry Research Centre, Edinburgh 9

(*Received* 19 *November* 1956)

Microanalyses of reducing sugars in avian bloods have been attempted many times with the general indication that the concentrations are markedly higher than those found in mammals. Most previous workers have used methods that are affected by *restreduktion* [non-carbohydrate blood components (cf. Herbert & Bourne, 1930)]. In a few instances 'fermentable sugar' has been estimated (Cassidy, Dworkin & Finney, 1926; Erlenbach, 1938; Fujita & Iwataki, 1931; Cavett, 1939). However, the addition of a washed yeast suspension to deproteinized blood, in which only few of the constituents have ever been identified, need not necessarily result in absorption of reducing sugars alone; other reducing components may be taken up by the yeast. Indeed, during fermentation, reducing substances can be formed [cf. Meyer (1913) and Merten (1938)].

The Folin & Wu (1919) method, as applied to the analysis of blood sugar in fowls, has been adversely criticized by Cassidy *et al.* (1926), Gulland & Peters (1930) and Fujita & Iwataki (1931). Ferricyanide methods, mainly that of Hagedorn & Jensen (1923), have also received adverse criticism by Gulland & Peters (1930), Erlenbach (1938) and Fujita & Iwataki (1931).

Few determinations of the blood sugar of fowls by titrimetric copper methods have been recorded. Strange, Dark & Ness (1955) have shown that both ferricyanide and copper reagents can be interfered with by amino acids as well as by creatine, uric acid, etc. and that Cd^{2+}, Ba^{2+} and Hg^{2+} will not satisfactorily remove these substances. These authors, likewise, found the copper reagents superior to ferricyanide.

Table 1 summarizes those papers known to the author where sufficient data have been given to allow comparison with the results of this paper. It is not claimed that this table is complete; because of indexing lacunae this field is difficult to search. The breeds of fowls, where known, are not stated in the table; there is no evidence so far of any influence of this factor on the blood sugar. It was therefore considered necessary, as a preliminary to future experimental work, that a search be made for a simple method which would determine the blood glucose in agreement with assays using D-glucose oxidase. The stabilized benzidine reagent of Jones & Pridham (1954) was found not to be affected by non-sugar components of three kinds of blood-'filtrates'; it gives results agreeing closely with blood-glucose determinations by means of D-glucose oxidase. The author is grateful to Professor A. St George Huggett, who drew his attention to the possibilities of the benzidine method (cf. Huggett, 1956).

METHODS

The birds used were Brown Leghorns taken either from pure lines or crosses from these lines, and were superficially healthy. No genetical influence on the blood-sugar levels was found. Unless fasted, the birds were allowed free access to the normal food used at this Centre.

This is a mash consisting of (in parts by weight) parings, 15·5; bran, 14·5; maize meal, 22·0; Sussex ground oats, 20·0; white fish meal, 9·5; dried unextracted brewer's yeast, 4·0; dried skimmed milk, 5·0; dried grass meal, 5·0; ground limestone, 1·0; common salt, 0·5; cod-liver oil (stabilized), 2·0; mineral mixture, 1·0. The last-named has the following composition (lb./approx. ton): exsiccated iron sulphate, 56; hydrated manganese sulphate, 124; limestone flour, 1399; sterilized feeding bone flour, 314; common salt, 336;

potassium iodide, 1½; cobalt sulphate, 1½; copper sulphate, 34. Mash is fed in roughly equal amounts with a grain mixture, consisting of kibbled maize, 33 lb.; whole wheat, 33 lb.; whole oat groats, 34 lb. The birds have *ad libitum* supplies of a mixture of flint grit and oyster-shell grit.

Blood sampling. (*a*) For large volumes, the animals were anaesthetized with veterinary Nembutal (45–60 mg.) injected into the alar vein. When anaesthesia was complete the abdomen was opened and blood collected directly from the heart. (*b*) Amounts of 1–5 ml. were taken by syringe from the alar vein. (*c*) As anticoagulants, citrate, heparin or sodium laminarin sulphate (O'Neill, 1955; O'Neill & Hawkins, 1955) were tried. Citrate (about 2 mg./ml.) was found to be satisfactory.

'Deproteinized' blood filtrates. Type A. Blood (1 vol.) was added to water (9 vol.) containing 0·24 mg. of acetic acid for each millilitre of blood (cf. Hunter, 1956). After mixing and laking, the whole was kept in a boiling-water bath, with frequent shaking, until coagulation was complete (5 min.), and filtered (Whatman no. 30 paper). Unless these filtrates were used immediately they were saturated with benzoic acid and stored at −20°.

Type B. The procedures of Somogyi (1945), with $ZnSO_4$ and $Ba(OH)_2$ in the cold, and of Fujita & Iwataki (1931), with $CdSO_4$ and NaOH in the hot, were followed exactly.

Type C. Blood (1 vol.) was added to 10% (w/v) trichloroacetic acid solution (9 vol.), and the mixture was shaken, kept for about ½ hr., and filtered through a Whatman no. 30 paper. This deproteinization is most convenient for routine work.

Detection of D-*glucose.* Paper chromatography of 80% ethanolic extracts of serum and paper electrophoresis in borate buffer of concentrated (A) filtrates showed no sugar other than glucose. 1:2-5:6-Di-*O*-*iso*propylidene D-glucofuranose (m.p. 110°), $[\alpha]_D - 12°$ ($CHCl_3$), was isolated by Bell's (1947) method from type A filtrates.

Measurements of reducing power. Titrimetric determinations were made on filtrates of types A and B by the 2 ml. scale copper method of Somogyi (1952); 0·0025 N-thiosulphate was satisfactory for the titrations.

Filtrates of types A and C were examined by the benzidine colorimetric method for aldoses (Jones & Pridham, 1954); with type C filtrates it was necessary for the appropriate amounts of trichloroacetic acid to be present in both blanks and standards, as the colours of water and trichloroacetic acid solutions are not spectrophotometrically comparable. Duplicate determinations invariably agreed so closely that single measurements are sufficient for routine analyses. With some bloods a slight precipitate, easily removed on the centrifuge, formed on heating with the benzidine reagent.

With both methods, standard glucose solutions prepared and stored as described by Bacon & Bell (1948) were invariably run concurrently with each set of analyses.

Table 1. *Summary of published values for the 'blood sugar' (mg./100 ml. of whole blood) in fowls*

Methods used. Type I: the Folin–Wu type (Folin & Wu, 1919; Folin, 1929; Folin & Malmros, 1929; Benedict, 1925 Rothberg & Evans, 1923). Type II: the Hagedorn–Jensen type (Hagedorn & Jensen, 1923; Fujita & Iwataki, 1931; Burr & Tanimoto, 1950; Kingsley & Reinhold, 1949). The last two are colorimetric. Type III: miscellaneous (Baudoin & Lewin, 1927; Somogyi, 1930, 1945; Shaffer & Hartman, 1921; Bertrand modified by Best, Hoet & Marks, 1926).

Sex	Condition	Methods (type)	Average values (range)	Individual values (range)	Authors
F.	Fed adult	I	139–279	91–333	1–9
		II	183–246	175–307	10–14
		III	197	175–225	16
			173	—	26
F.	Fasted adult	I	186 (155*)	—	3
		II	152–182	150–290	13, 14, 15
		III	162	148–201	16
M.	Fed adult	I	161–203 (170*)	148–260	3, 16, 17
		II	188–218	143–236	12. 14, 18, 19
M.	Fasted adult	I	180 (150*)	178–181	3
		II	172–180	78–232	18, 20
Mixed	Fed, 1–4 months	I	188†	—	25
		III	162–228	146–278	21–24
Mixed	Fasted, 1–4 months	I	148–169	—	25
		III	182–195	175–206	22, 23

* 'Fermentable reduction'. † 424 birds, average increased with age.

Authors

(1) Scheunert & Pelchrizm (1923)
(2) Thompson & Carr (1923)
(3) Cassidy *et al.* (1926)
(4) Hayden (1929)
(5) Horvath (1930*a*, *b*)
(6) Hayden & Sampson (1931)
(7) Dyer & Roe (1934)
(8) Russell & Weber (1934)
(9) Bronkhorst & Hall (1935)
(10) Schwartz & Heinrich (1928)
(11) Fukita & Iwataki (1931)
(12) Erlenbach (1938)
(13) Murray & Rosenberg (1953)
(14) Sturkie (1955)
(15) Macowan & Magee (1932)
(16) Rogement (1930)
(17) Gonzago (1935)
(18) Krasnianski & Dsikowski (1931)
(19) Burrows, Fritz & Titus (1935)
(20) Henry, Magee & Reid (1934)
(21) Hogan, Shrewsbury & Kempton (1928)
(22) Corkill (1930)
(23) Golden & Long (1942)
(24) Hsu & Combs (1952*a*, *b*)
(25) Opdyke (1942)
(26) Tapper & Kare (1956)

Determination of D-*glucose by* D-*glucose oxidase* (cf. Keilin & Hartree (1948) and Bell & Manners (1952)). Samples of five large-scale type A filtrates were concentrated under reduced pressure (bath temp. below 30°) to volumes corresponding to those of the original bloods. Portions (1 ml.) were incubated in Warburg manometer flasks with catalase (1 ml.) [300 mg. of commercial beef enzyme (L. Light and Co. Ltd.) in 15 ml. of M/30 phosphate buffer, pH 6·5] and glucose oxidase solution (0·2 ml. initially in side bulb). The very large amount of crude catalase was required to produce a maximal oxygen uptake; the reason for this is unknown. The glucose oxidase was commercial material (Takamine Laboratories Inc.); 15 mg. was suspended in 10 ml. of M/30 phosphate buffer (pH 6·5), solid removed on the centrifuge and the clear, supernatant fluid stored at approx. 2°. Controls were run against the catalase alone, and against 2 mg. amounts of D-glucose. The oxygen uptakes observed in three experiments were between 96·5 and 98·9% of the theoretical 124·4 μl.

De-ionization of deproteinized filtrates (type A). As found by Strange *et al.* (1955), Amberlites IR-4B(OH) and IR-120(H) in equal weights did not remove glucose from solution. Experiments showed that shaking 1 vol. of a 1:10 filtrate with one-twentieth of its weight of each resin for 1 hr. removed practically all the non-protein nitrogen and the unknown substances which interfered with cuprimetric determination of the sugar.

Non-protein nitrogen. The procedure of Chibnall, Rees & Williams (1943) was followed.

RESULTS

Preliminary examination of methods. Numerous experiments on blood filtrates of types A and B indicated that the cuprimetric method was not satisfactory. It certainly could not be applied to type A filtrates since the results, unexpectedly, were often very low (cf. Strange *et al.* 1955); the Somogyi and Fujita–Iwataki deproteinization methods gave filtrates containing more 'glucose' than could be found by D-glucose oxidase. On the other hand, the results of the benzidine colorimetric method were consistently in agreement with the true glucose, both when used directly on type A filtrates, or after deionization. The cuprimetric method used on deionized type A filtrates also gave satisfactory agreement. Despite the numerous possible sources of manipulative error, maximum differences of approximately 5% between the glucose oxidase results and those of the benzidine method were the highest observed. It was therefore concluded that this latter easy and rapid technique was the method of choice for the subsequent survey of blood-sugar values. The blank is colourless and the reaction colour is stable for over 24 hr. Table 2 summarizes one of a number of preliminary experiments where the non-protein nitrogen of the various blood filtrates was examined as well as the apparent sugar contents.

To check that trichloroacetic acid (type C filtrates) did not affect the benzidine determinations, two sets of parallel measurements were done on types A and C filtrates made from the same blood samples. The results are given in Table 3; it will be seen that type C filtrates are satisfactory for the determination of glucose by the benzidine method.

Table 2. *Comparison of copper and benzidine methods with* D-*glucose oxidase method*

Three different procedures for deproteinization, and resin deionization, were employed. Glucose and non-protein nitrogen (N.P.N.) were determined as mg./100 ml. of whole blood (mean of duplicate and triplicate determinations), on five cocks.

Type A filtrates					
D-Glucose oxidase determination	275	174	179	230	173
Benzidine determination	264	169	179	212	177
Somogyi determination	160	75	85	175	80
N.P.N.	51·8	52·6	69·5	64·3	47·0
Type A filtrates, deionized					
Benzidine determination	260	169	178	218	184
Somogyi determination	253	172	176	219	186
N.P.N.	3·9	3·2	1·5	5·9	0
Somogyi filtrate					
Somogyi determination	319	209	224	274	195
N.P.N.	24·6	35·8	32·8	40·0	35·8
Fujita–Iwataki filtrate					
Somogyi determination	298	214	219	247	170
N.P.N.	30·4	38·7	44·6	35·5	36·2

Table 3. *Comparison of glucose (mg./100 ml. of whole blood) determinations on type A and type C filtrates*

Blood sample ...	1	2	3	4	5	6
Type A filtrate	170	80	137	147	130	97
	170	77	138	147	130	95
Type C filtrate	175	75	137	—	126	93
	175	77	—	146	137	—

Table 4. *Blood-glucose levels in fowls of various ages*

These are mainly C-type filtrates. A few determinations were done on A filtrates and some on both A and C types.

Group	Sex	No. in group	Age (months)	Fed or fasting	Individual values for glucose (mg./100 ml. of whole blood)	Average
1	F.	16	4–6	Fed	195, 196, 200, 213, 213, 215, 215, 215, 218, 220, 225, 226, 235, 235, 243, 243	219·19
2	F.	15	12–23	Fed	166, 182, 190*, 192*, 195, 198*, 200, 206, 207, 210*, 212, 212, 214*, 217, 225*	201·73
3	F.	20	24–35	Fed	168*, 175, 187*, 192, 192, 192, 193, 194, 196*, 208, 210, 212, 212*, 213, 218*, 218, 222*, 222, 222*, 230	203·80
4	F.	15	36–60	Fed	148, 158*, 164, 170, 170, 178, 185, 187, 188, 195, 197, 205, 215, 218, 234*	187·47
5	M.	9	4–6	Fed	177, 190, 190, 200, 201, 210, 213, 215, 219	201·67
6	M.	7	12–24	Fed	134, 147, 150, 155, 165, 171, 184	158·00
7	M.	5	12–24	Fasted, 48 hr.	77, 95, 130, 138, 147	117·40

* Denotes values for laying hens.

The groups are clearly heterogeneous, only groups 2 and 3 showing no significant difference between them. *t*-Tests show the following differences to be significant at the 1% level: males (groups 5 and 6) have a lower blood glucose than females (groups 1 and 2); young females (group 1 against group 2 and groups 2 and 3 against group 4) have a higher blood glucose than older females: similarly, young males (group 5) have a significantly higher blood glucose than older males (group 6). Fasted males (group 7) have a lower blood glucose than unfasted males (group 6), the *t* value lying between the 1 and 2% levels of probability. There is no difference between the blood-glucose levels of laying (mean 204·15) and non-laying (mean 202·18) hens.

Blood-glucose levels of fowls according to sex and age. The results from seventy birds are in Table 4. The average glucose level tends to be lower in males than in females, and in both sexes a decrease accompanies ageing. In cocks, fasting for 48 hr. lowered the level considerably below that of the fed animal. In the hen, laying did not appear to be accompanied by any characteristic blood-glucose level.

DISCUSSION

As Sturkie (1954, 1955) has pointed out, there are considerable variations in the values reported by different workers for the reducing sugar of fowl's blood (cf. Table 1). In some instances, the value of exhaustive surveys is greatly lessened because one or more factors are omitted from the data. For example age, condition and sex may not be taken into consideration, despite early indications of their importance (e.g. Gioja, 1912).

Since we know very little about the *restreduktion* materials in avian blood and how they affect the reagents employed in reducing-power determinations, it seems neither possible nor profitable to speculate about the wide scatter of the individual and average values of Table 1. The results presented above lead to the following conclusions: (1) Fowl blood has a much higher true glucose level than mammalian blood; no sugar other than D-glucose could be found. (2) In both sexes, the glucose level tends to decrease with age, as previously suggested by Gioja (1912), Heller & Pursell (1937) and Batt (1939). This may be due to a slow rise in erythrocyte volume with age, as indicated by unpublished observations in this Laboratory. Cocks tend to have a lower blood sugar than hens. This is partly, if not wholly, due to the higher erythrocyte volume in the male (Newell & Shaffner, 1950). Unpublished results from this Laboratory have shown that fowl erythrocytes contain little or no glucose (cf. Tapper & Kare, 1956). (3) Ovulation, or laying, cannot be correlated with the glucose level of the hen's blood, in agreement with Heller & Pursell (1937). (4) Although previous workers have used methods that do not solely determine reducing sugars, their findings are none the less of the same general order as those with the benzidine method. This may be due to 'compensation' by non-sugar blood constituents, some of which may cause 'over-reduction' and others 'under-reduction'. In our experience, blood filtrates made by several methods contain ionizable substances which can cause over- or under-reduction of Somogyi's (1952) reagent.

SUMMARY

1. Use of the stabilized benzidine reagent of Jones & Pridham (1954) on suitable filtrates of fowl blood enables the D-glucose content to be determined colorimetrically, as checked by D-glucose oxidase. The method is simple and the colour produced is stable for at least 24 hr.

2. The average blood-glucose levels of groups of fed hens falls from approximately 220 mg./100 ml.

at 4–6 months to approximately 190 mg./100 ml. at 4–6 years.

3. The average blood glucose of cocks is lower than that of hens, probably owing to greater erythrocyte volumes in the former. It falls from approximately 200 mg./100 ml. at 4–6 months to approximately 160 mg./100 ml. at 1–2 years, and is considerably lowered by fasting.

4. Blood 'filtrates' prepared according to both Somogyi (1945) and Fujita & Iwataki (1931) gave high results with Somogyi's (1952) reagent.

5. No sugars other than D-glucose could be detected in fowl blood.

6. Previous work in this field is summarized and briefly criticized.

REFERENCES

Bacon, J. S. D. & Bell, D. J. (1948). *Biochem. J.* **42**, 397.
Batt, H. T. (1939). *Amer. J. Physiol.* **126**, 429.
Baudoin, A. & Lewin, J. (1927). *Bull. Soc. Chim. biol., Paris*, **9**, 280.
Bell, D. J. (1947). *J. chem. Soc.* p. 1461.
Bell, D. J. & Manners, D. J. (1952). *J. chem. Soc.* p. 3461.
Benedict, S. R. (1925). *J. biol. Chem.* **44**, 207.
Best, C., Hoet, J. P. & Marks, H. P. (1926). *Proc. Roy. Soc.* B, **100**, 32.
Bronkhorst, J. J. & Hall, G. O. (1935). *Poultry Sci.* **14**, 42.
Burr, G. O. & Tanimoto, T. (1950). *Fed. Proc.* **9**, 157.
Burrows, W. H., Fritz, J. C. & Titus, W. H. (1935). *J. biol. Chem.* **110**, 39.
Cassidy, G. J., Dworkin, S. & Finney, W. H. (1926). *Amer. J. Physiol.* **75**, 609.
Cavett, J. W. (1939). *Poultry Sci.* **18**, 150.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Corkill, B. (1930). *Biochem. J.* **24**, 779.
Dyer, H. M. & Roe, J. H. (1934). *J. Nutr.* **7**, 623.
Erlenbach, F. (1938). *Z. vergl. Physiol.* **26**, 121.
Folin, O. (1929). *J. biol. Chem.* **82**, 83.
Folin, O. & Malmros, H. (1929). *J. biol. Chem.* **83**, 115.
Folin, O. & Wu, H. (1919). *J. biol. Chem.* **38**, 81.
Fujita, A. & Iwataki, D. (1931). *Biochem. Z.* **242**, 43.
Gioja, J. (1912). *C.R. Soc. Biol., Paris*, **73**, 102.
Golden, W. R. C. & Long, C. N. H. (1942). *Endocrinology*, **30**, 675.
Gonzago, A. (1935). *Rep. N.Y. St. vet. Coll.* 1933–34, p. 53.
Gulland, J. M. & Peters, R. A. (1930). *Biochem. J.* **24**, 91.
Hagedorn, J. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
Hayden, C. E. (1929). *Rep. N.Y. St. vet. Coll.* 1927–28, p. 213.
Hayden, C. E. & Sampson, J. E. (1931). *Rep. N.Y. St. vet. Coll.* 1929–30, p. 174.
Heller, V. G. & Pursell, L. (1937). *J. biol. Chem.* **118**, 549.
Henry, K. M., Magee, H. E. & Reid, E. (1934). *J. exp. Biol.* **11**, 58.
Herbert, F. K. & Bourne, M. C. (1930). *Biochem. J.* **24**, 299.
Hogan, A. G., Shrewsbury, C. L. & Kempton, H. L. (1928). *Res. Bull. Mo. agric. exp. Sta.* no. 124.
Horvath, A. A. (1930*a*). *Poultry Sci.* **9**, 313.
Horvath, A. A. (1930*b*). *Amer. J. Physiol.* **94**, 65.
Hsu, P. T. & Combs, G. F. (1952*a*). *Arch. Biochem. Biophys.* **38**, 29.
Hsu, P. T. & Combs, G. F. (1952*b*). *J. Nutr.* **47**, 73.
Huggett, A. St G. (1956). *J. Physiol.* **132**, 3*P*.
Hunter, G. (1956). *Biochem. J.* **62**, 28P.
Jones, J. K. N. & Pridham, J. B. (1954). *Biochem. J.* **58**, 288.
Keilin, D. & Hartree, E. F. (1948). *Biochem. J.* **42**, 230.
Kingsley, G. R. & Reinhold, J. G. (1949). *J. Lab. clin. Med.* **34**, 713.
Krasnianski, L. & Dsikowski, W. (1931). *Biochem. Z.* **237**, 282.
Macowan, M. & Magee, H. E. (1932). *Quart. J. exp. Physiol.* **21**, 276.
Merten, R. (1938). *Biochem. Z.* **297**, 304.
Meyer, P. (1913). *Biochem. Z.* **50**, 362.
Murray, H. C. & Rosenberg, M. M. (1953). *Poultry Sci.* **32**, 805.
Newell, G. W. & Shaffner, C. S. (1950). *Poultry Sci.* **29**, 78.
O'Neill, A. N. (1955). *Canad. J. Chem.* **33**, 1097.
O'Neill, A. N. & Hawkins, W. W. (1955). *Canad. J. Biochem. Physiol.* **33**, 545.
Opdyke, D. F. (1942). *Endocrinology*, **31**, 363.
Rogement, L. (1930). *C.R. Soc. Biol., Paris*, **104**, 154, 256.
Rothberg, V. E. & Evans, F. A. (1923). *J. biol. Chem.* **58**, 431.
Russell, W. C. & Weber, A. L. (1934). *Poultry Sci.* **13**, 376.
Scheunert, A. & Pelchrizm, H. von (1923). *Biochem. Z.* **139**, 17.
Schwartz, K. & Heinrich, K. (1928). *Biochem. Z.* **194**, 346.
Shaffer, P. A. & Hartman, A. F. (1921). *J. biol. Chem.* **45**, 344.
Somogyi, M. (1930). *J. biol. Chem.* **86**, 655.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
Strange, R. E., Dark, F. A. & Ness, A. G. (1955). *Biochem. J.* **59**, 172.
Sturkie, P. D. (1954). *Avian physiology*. Ithaca: Comstock Publ. Associates.
Sturkie, P. D. (1955). *Endocrinology*, **56**, 575.
Tapper, D. N. & Kare, M. R. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 120.
Thompson, T. J. & Carr, I. L. (1923). *Biochem. J.* **17**, 373.

The Mechanism of the Reaction Between Di-(2-chloroethyl) Sulphone (Mustard-Gas Sulphone) and Amino Acids

By G. E. Francis, Denise E. Richards and A. Wormall
Department of Biochemistry, The Medical College of St Bartholomew's Hospital, London, E.C. 1

(*Received* 24 *September* 1956)

It has been demonstrated that both di-(2-chloroethyl) sulphone (mustard-gas sulphone, HO_2) and divinyl sulphone react with proteins and with the amino groups of α-amino acids to give derivatives of 1:4-thiazan 1:1-dioxide, the products obtained in each case being the same, whichever sulphone is used (Boursnell, Francis & Wormall, 1946*a*, *c*). A. H. Ford-Moore and A. H. Ford-Moore & A. G. Lidstone (personal communications) have also shown that HO_2 in alkaline solution loses HCl to give 2-chloroethyl vinyl sulphone, and then divinyl sulphone, and have suggested that this reaction precedes the reaction with amino groups. The finding that HO_2, which had previously been treated at 37° with bicarbonate buffer at pH 7·4 until most of its chlorine had become ionized, reacts with amino acids more rapidly than does HO_2, but less rapidly than does divinyl sulphone (Boursnell *et al.* 1946*a*), also lends support to this view.

The nucleophilic substitution reaction between a chloroethyl group and an amino group involves first the formation of a carbonium ion:

$$X\cdot CH_2\cdot CH_2\cdot Cl \rightarrow X\cdot CH_2\cdot CH_2^+ + Cl^-.$$

The carbonium ion may then react in one of two ways, either by direct condensation with the amino group and expulsion of H^+ from the latter:

$$X\cdot CH_2\cdot CH_2^+ + HNR_2 \rightarrow X\cdot CH_2\cdot CH_2\cdot NR_2 + H^+ \quad (1)$$

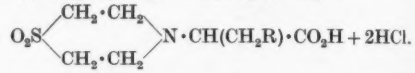
or by liberation of H^+ with formation of a vinyl group, followed by addition of the amino compound to the ethylenic bond:

$$X\cdot CH_2\cdot CH_2^+ \rightarrow X\cdot CH{:}CH_2 + H^+$$
$$X\cdot CH{:}CH_2 + HNR_2 \rightarrow X\cdot CH_2\cdot CH_2\cdot NR_2. \quad (2)$$

If mechanism (1) is correct, both hydrogen atoms on the carbon atom in the β-position to the nitrogen in the product would be derived from the chloroethyl group. If mechanism (2) is correct, one of these hydrogen atoms would be derived from the chloroethyl group and one from the amino group.

In order to determine to what extent a preliminary formation of vinyl groups occurs in the reaction between HO_2 and amino acids we have prepared the condensation products of two amino acids, α-alanine and β-phenylalanine, with HO_2 labelled with deuterium on all eight of its hydrogen atoms. The over-all reaction can be represented:

$$O_2S(CH_2\cdot CH_2Cl)_2 + H_2N\cdot CH(CH_2R)\cdot CO_2H \rightarrow$$



$$+ 2HCl.$$

If this reaction follows mechanism (1) exclusively, the products should each contain eight labelled hydrogen atoms/molecule, but if mechanism (2) operates exclusively, only six. A result between six and eight would indicate simultaneous operation of both mechanisms.

EXPERIMENTAL

'Deutero'-mustard-gas sulphone. This was prepared from deutero-mustard gas (Boursnell, Francis & Wormall, 1946*b*) by oxidation with H_2O_2–acetic acid as described by Francis, Mulligan & Wormall (1955). The product was diluted with exactly 49 times its weight of ordinary HO_2 and recrystallized from ethanol.

HO_2–amino acid derivatives. These were prepared essentially as described previously (Boursnell *et al.* 1946*a*). Alanine (0·01 mole) and β-phenylalanine (0·0075 mole) were each treated with an equimolar amount of the deutero-HO_2 in the presence of three equivalents of M-$NaHCO_3$. The mixtures were shaken at 37° for 3 hr. and left overnight at room temperature, the pH being maintained at about 7·5 (phenol red internal indicator) by the addition of drops of N-NaOH as required. The alanine derivative was isolated *via* the copper salt (Boursnell *et al.* 1946*a*) and the phenylalanine derivative by acidification with HCl. The alanine derivative was recrystallized from 50% (v/v) ethanol (17 ml.) and the phenylalanine derivative from boiling water (300 ml.).

For reasons given below the possibility was considered that the phenylalanine–sulphone derivative, like the corresponding glycine derivative, contained a firmly bound molecule of water of crystallization (Boursnell *et al.* 1946*a*). Analysis of the preparation (Found: C, 55·0; H, 6·1; N, 4·9; S, 11·2. $C_{13}H_{17}NSO_4$ requires C, 55·1; H, 6·05; N, 4·95; S, 11·3%) and the fact that there was no significant loss of weight when it was dried at 100° showed, however, that the phenylalanine–sulphone was free from water of crystallization.

Determination of deuterium. Samples (30–50 mg.) of the two amino acid derivatives and of the deutero-HO_2 (diluted and recrystallized with an equal weight of ordinary HO_2) were covered with freshly ignited CuO and burnt in dry O_2 in a silica combustion tube filled with CuO (wire, form M.A.R.).

Table 1. *Comparison of deuterium contents of deuterodi-(2-chloroethyl) sulphone and its condensation products with amino acids*

Compound	Mol. formula	Atom % excess of deuterium*	No. of 'sulphone hydrogens'/molecule†
Di-(2-chloroethyl) sulphone	$C_4H_8SO_2Cl_2$	1·84±0·021 (3) 1·80–1·88	8
'Alanine sulphone' (α-thiazan-4-ylpropionic acid 1:1-dioxide)	$C_7H_{13}NSO_4$	0·87±0·011 (7) 0·83–0·91	6·2
'Phenylalanine sulphone' (β-phenyl-α-thiazan-4-ylpropionic acid 1:1-dioxide)	$C_{13}H_{17}NSO_4$	0·58±0·0074 (8) 0·56–0·61	5·4

* The mean values, S.E., number of determinations (in parentheses) and extreme range of values are given in each case.

† Calculated by multiplying the ratio (atom % excess of D in compound)/(atom % excess of D in HO_2) by the number of H atoms/molecule of compound.

The water formed was collected in a trap cooled in solid CO_2–ethanol and redistilled (cf. Graff & Rittenberg, 1952). The water was reduced to H_2 by heating with Zn dust at 400° (Dubbs, 1953), and the deuterium content of the gas was determined on a Consolidated-Nier Isotope Ratio Mass Spectrometer, calibrated with H_2 samples of known deuterium content.

RESULTS

The results of the deuterium determinations on the HO_2 and the two HO_2-amino acid compounds are shown in Table 1. It is always difficult to obtain closely concordant results in repeated deuterium determinations on an organic compound, owing to the relatively large errors introduced by very small amounts of moisture contamination occurring at any stage during the manipulations, but the figures given for atom percentage excess deuterium are the means of a number of determinations, the spread of individual results and the standard error being given in the table in each case.

DISCUSSION

The results of the deuterium determinations of the two amino acid–HO_2 condensation products preclude the possibility of any significant reaction proceeding via a direct nucleophilic substitution mechanism. The result with the alanine derivative is just within the limits of experimental error for a mechanism proceeding exclusively via the formation of two vinyl groups. With the phenylalanine derivative the result is lower than that required by this mechanism by an amount slightly greater than can be accounted for by experimental error in the determinations. Since the presence of water of crystallization in the compound is precluded by the elementary analyses, it appears probable that there was a slight loss of the deuterium label during this particular preparation. The loss of deuterium from compounds of this type by an exchange reaction seems unlikely, but it may be significant that this compound, unlike the alanine–sulphone, was at one stage of its preparation in contact with a strongly acid medium. Any exchange of deuterium which did occur must, however, have been very small, otherwise the deuterium content would have been considerably lower. It is highly improbable that an exchange reaction could have accounted for the result in both cases being so close to six, if, in fact, significantly more than six of the hydrogen atoms were derived from the sulphone.

It therefore appears that, at least with these two amino acids, the reaction proceeds exclusively via the formation of two vinyl groups, followed by addition of the amine to form the cyclic compound. Since HO_2 reacts with proteins chiefly as a result of condensation with the free amino groups of the protein (Banks, Boursnell, Francis, Hopwood & Wormall, 1946), it seems logical to conclude that in this reaction also the condensation occurs only after the HO_2 has been converted into divinyl sulphone.

SUMMARY

1. α-Thiazan-4-ylpropionic acid 1:1-dioxide and β-phenyl-α-thiazan-4-ylpropionic acid 1:1-dioxide labelled with deuterium in the thiazan ring have been prepared by treating alanine and β-phenylalanine respectively with di-(2-chlorotetradeuteroethyl) sulphone in aqueous solution at 37° and pH 7–8.

2. Deuterium determinations on these two compounds show that in each case six hydrogen atoms/molecule carry a deuterium label.

3. From this result, and other evidence, it is concluded that the reaction between di-(2-chloroethyl)

sulphone and the amino groups of α-amino acids proceeds exclusively *via* the formation of two vinyl groups in the sulphone, followed by the addition of the amine to the ethylenic bonds.

We are indebted to the British Empire Cancer Campaign for grants which have partly covered the cost of these investigations. We are also grateful to Dr T. E. Banks for advice and assistance in the use and maintenance of the mass spectrometer and to Dr V. C. E. Burnop for help with one stage of the synthesis of deutero-mustard-gas sulphone and also for his assistance in some of the early investigations. The microanalyses were carried out by Miss J. Cuckney and the staff of the Microanalytical Laboratories of the Imperial College.

REFERENCES

Banks, T. E., Boursnell, J. C., Francis, G. E., Hopwood, F. L. & Wormall, A. (1946). *Biochem. J.* **40**, 745.
Boursnell, J. C., Francis, G. E. & Wormall, A. (1946*a*). *Biochem. J.* **40**, 737.
Bournsell, J. C., Francis, G. E. & Wormall, A. (1946*b*). *Biochem. J.* **40**, 743.
Boursnell, J. C., Francis, G. E. & Wormall, A. (1946*c*). *Biochem. J.* **40**, 768.
Dubbs, C. A. (1953). *Analyt. Chem.* **25**, 828.
Francis, G. E., Mulligan, W. & Wormall, A. (1955). *Biochem. J.* **60**, 118.
Graff, J. & Rittenberg, D. (1952). *Analyt. Chem.* **24**, 878.

The Quantitative Estimation of Pyruvic and α-Oxoglutaric Acids by Paper Chromatography in Blood, Urine and Cerebrospinal Fluid

By B. McARDLE
Member of Scientific Staff, Medical Research Council, Guy's Hospital Medical School, London, S.E. 1

(*Received* 10 *September* 1956)

It is now recognized that the estimation of pyruvate by the Friedemann & Haugen method (1943) carries with it an element of uncertainty owing to the non-specific nature of this technique. This lack of specificity may well be unimportant for estimations in human blood and cerebrospinal fluid, in which, under normal conditions, other α-keto acids are present in small or negligible amounts, but if a clearer insight is to be gained into the disturbances of the Krebs cycle in disease a more specific determination of the individual α-keto acids is required. This is especially true if α-oxoglutaric and other α-keto acids are present in significant amounts as, for example, in the blood of certain animals such as the rat, in certain tissues and in urine. A specific procedure involving the formation of the 2:4-dinitrophenylhydrazones of the α-keto acids and their separation by paper chromatography was devised by Cavallini, Frontali & Toschi (1949*a*, *b*), and since then a number of modifications of their method have been published. These methods, however, with few exceptions have been tedious and time-consuming, and in few is it possible to assess their accuracy. The present method has been developed from that of El Hawary & Thompson (1953) and has given satisfactory results over three years. The number of extractions has been greatly reduced, and the need for evaporating the extracted hydrazones to dryness eliminated. The addition of ethanol to the ethyl acetate used for extracting the hydrazones has further shortened the procedure by greatly reducing interference due to emulsions, which are particularly apt to develop during blood determinations.

METHOD

Reagents. 2:4-Dinitrophenylhydrazine, 0·2% in 2N-HCl; ethanol, absolute; ethyl acetate (A.R.); ammonium hydroxide, 2·5N; metaphosphoric acid, 5 and 10% (w/v) freshly prepared from a stock 50% solution; solvent mixture: *n*-butanol–ethanol–N-NH_3 soln. (13:2:5, by vol.); sodium hydroxide–sodium carbonate solution, freshly prepared by mixing equal parts of 2·5N-NaOH (A.R.) and 10% (w/v) Na_2CO_3 (A.R.).

Blood. Immediately after withdrawal 5–7 ml. of blood is mixed with 20 ml. of 5% metaphosphoric acid in a weighed tube. After a few minutes the mixture is centrifuged.

Urine. If the specific gravity is 1·02 or over, 3 ml. of urine is added to 13 ml. of 5% metaphosphoric acid. Below this specific gravity, increasing quantities of urine are added to decreasing amounts of more concentrated metaphosphoric acid, the amount of urine depending on the specific gravity, and the final concentration of the acid being not less than 4%. If the urine contains protein this must first be precipitated and removed by centrifuging.

Cerebrospinal fluid. A volume (3 ml.) is mixed with 14 ml. of 5% metaphosphoric acid, and centrifuged.

Preparation and extraction of keto acid hydrazones. A volume (2 ml.) of the 2:4-dinitrophenylhydrazine in 2N-HCl is added to 16 ml. of the centrifugate or, with protein-free urine, of the clear solution, and the mixture is incubated for 20 min. at 38°. The mixture is then shaken in a 30 ml. separating funnel with 5 ml. of ethyl acetate and 5 ml. of ethanol for 0·75 min., and the layers are allowed to separate. The aqueous phase is re-extracted successively with 5 ml. and then with 1 ml. of ethyl acetate. With blood filtrates some degree of emulsification usually interferes with the complete separation of the two phases during the second and third extractions. If this occurs the emulsion is added to the first ethyl acetate extract and centrifuged at 2000 rev./min. for 1–2 min. The aqueous layer is returned to the separating funnel and extracted for the third time. The contents of the

separating funnel are then transferred to a centrifuge tube and spun for 1–2 min. The aqueous layer is sucked off and the ethyl acetate layer is added to the earlier extracts. The combined ethyl acetate phase is then washed by shaking with 20 ml. of water made faintly acid with 1–2 drops of N-HCl. If necessary, it is centrifuged for 1 min. and all the aqueous layer is carefully sucked off and discarded. Aqueous 2N-NH_3 soln. (1·5 ml.) is then added to the ethyl acetate and oxygen or compressed air is bubbled through for 2·5 min. When separation is complete, the aqueous layer is pipetted into a suitable tube.

Chromatographic procedure. From 0·1 to 0·6 ml. of the final extract, depending on the expected keto acid content, is then placed on a strip of Whatman no. 3MM filter paper from a micrometer syringe fitted with a stainless-steel hypodermic needle having a plastic mount (Ballbrook Surgical Instrument Co. Ltd.). This type of needle is not attacked by ammonia. For urine 0·2 ml. is usually ample, but for blood and cerebrospinal fluid 0·4 and 0·6 ml. respectively are taken because of the low α-oxoglutarate levels. Much time and trouble are saved by placing two spots on the strip simultaneously from two Agla syringes, a single micrometer actuated by a suitably geared motor being used, which stops automatically when the required volume has been delivered. At the same time air from a hair dryer at a temperature not exceeding 40° is directed on to the spots. The paper is hung in a sealed-glass tank, and the solvent is allowed to ascend for about 16 hr. The solvent mixture used by El Hawary & Thompson (1953), *n*-butanol–ethanol–0·5N-NH_3 soln. (7:1:2), gives a wider separation of the spots between α-oxoglutarate and pyruvate, but this is at the expense of any hydrazones with a lower R_F value than α-oxoglutarate. It is thought that the solvent with the higher ammonia content used in the present work is more generally useful.

Identification. The paper strips are dried and the areas occupied by the hydrazones outlined with pencil, preferably under ultraviolet light. Although the sequence of the hydrazones on the paper is the same whether run in solvent containing 0·5 or N-NH_3 soln., the R_F values differ; α-oxoglutarate hydrazone has an R_F value, when run in the N-NH_3 soln. solvent, of approx. 0·28, the main pyruvate spot about 0·5 and the other pyruvate spot about 0·67. The spot of the hydrazone of acetoacetate, R_F about 0·57, is not as compact as those of pyruvate and α-oxoglutarate, but, although the upper border is rather indefinite, the lower edge is usually well defined. Quantitative measurement of acetoacetate has not been attempted since the proportion of the acid finally appearing as a hydrazone spot is very low. Thus 10 mg. of acetoacetic acid/100 ml. gives the same final colour intensity as 0·22 mg. of pyruvic acid/100 ml. Moreover, since the acetoacetate hydrazone spot is separated from that of pyruvate, small amounts of acetoacetate do not interfere with the quantitative determination of pyruvate. If, however, large amounts of this acid might be expected to be present, it is probably wise to destroy the acetoacetate hydrazone either by conversion into the fast-running acetone hydrazone (R_F 0·95) by heating the dried spot on the paper before chromatography in an oven at 60° for 20 min., or by adding 1 ml. of a 2% (w/v) solution of *p*-dimethylaminobenzaldehyde in 1·3N-HCl 3 min. before the first ethyl acetate extraction. Preliminary observations have shown that the first procedure destroys about 90% of the acetoacetic acid hydrazone but leaves the α-oxoglutarate hydrazone unaffected and only causes a loss of about 5% in the pyruvate hydrazone; with the second procedure 3 mg. amounts of acetoacetate added to blood filtrates are completely removed, but there is a rather larger loss of pyruvate (17%) and α-oxoglutarate (9%) hydrazones. Introduced into the Friedemann & Haugen method when ketonaemic blood is analysed, the second procedure reduces the increment in pyruvate estimation resulting from an acetoacetate concentration of 100 mg./100 ml. to 0·02 mg./100 ml. However, in these circumstances determinations of standard pyruvate and α-oxoglutarate solutions show a constant loss of 13 and 8% respectively. Allowance for this loss of pyruvate must therefore be made in the calculation.

The chromatograms of the more concentrated urines may show small amounts of unidentified substances absorbing ultraviolet light; these do not interfere with the identification of the pyruvate or α-oxoglutarate hydrazones. One of these spots has an R_F value about 0·31 and another about 0·37.

Elution and quantitative estimation. The area of paper occupied by the given hydrazone is cut into small pieces and placed in 5 ml. of the Na_2CO_3–NaOH mixture and the paper is ground with a glass rod until it has disintegrated. The mixture is then poured into a sintered-glass micro funnel (porosity 3), into which a small disk of filter paper (Whatman no. 42) has previously been inserted, and filtered under pressure into a small test tube. The coloured solution is refiltered through the same funnel. A solution blank is similarly filtered. Fifteen minutes after the addition of the alkali to the paper the red colour is measured in a 2 cm. cuvette with an Ilford Spectrum Violet 601 filter, and compared with a standard curve derived by eluting known amounts of the corresponding keto acid hydrazones from paper without subjecting them to chromatography. The optical density of the solution blank is also determined and a small correction added for the paper blank. For pyruvate spots this is 0·003 and for the smaller α-oxoglutarate spots 0·002. These values are the means of many estimations obtained by eluting, in the absence of added hydrazones, paper that has been treated overnight in the solvent mixture.

Colorimetric estimation of keto acids. The method of Friedemann & Haugen (1943) was also used for estimating the keto acids in blood. Ethyl acetate was used as solvent.

RESULTS

The extraction and chromatographic procedures result in some loss of α-keto acid hydrazones. The loss is greater for pyruvate than for α-oxoglutarate. The extent and constancy of the loss has been determined by comparing the final colour intensities given by known amounts of pyruvate and α-oxoglutarate with that of comparable amounts of synthetic hydrazones spotted on paper and immediately eluted. In thirty-five determinations of solutions containing 24–60 μg. of pyruvate/16 ml. the mean recovery was 64·3% (S.D. 2·81). The corresponding value for α-oxoglutarate in thirty-five determinations of solutions containing 20–60 μg./16 ml. was 80·9% (S.D. 2·04). Part of this loss is due to the slight increase in the volume of the NH_3 soln. phase, caused by the solubility of ethyl

acetate in the aqueous phase. Thus the 1·5 ml. of NH_3 soln. originally added increased to 1·6 ml. or over after mixing of the phases. This increase in volume is not allowed for in the calculation and leads to an apparent loss of hydrazone. A further source of loss was the incomplete (88 %) extraction of the hydrazones into the relatively small quantity of NH_3 soln. used; larger volumes of NH_3 soln. have, however, not given sufficiently greater extraction to offset the resulting decrease in concentration.

The mean values for recovery given above have been used in calculating all pyruvate and α-oxoglutarate values. The accuracy of the method in this series of thirty-five estimations is given in Table 1. Results for the recovery of 30 μg. amounts of pyruvate and α-oxoglutarate added to the deproteinized filtrates of blood, or to diluted urine, are given in Table 2.

As a further check on the method a comparison has been made, in 100 determinations on metaphosphoric acid filtrates of human blood, between the α-keto acid content as estimated by the Friedemann & Haugen method and the combined pyruvate and α-oxoglutarate content (expressed as pyruvate), determined by the chromatographic technique. The results are set out in Table 3. Good agreement was found over a wide range of pyruvate values, in specimens taken after fasting, after strenuous exercise, or after the ingestion of glucose both in normal subjects and in diseased states. The mean level of α-oxoglutarate in this series was 0·17 mg./100 g. (S.D. 0·06). In general the chromatographic values tended to be slightly lower than the values obtained by the Friedemann & Haugen method. This difference is certainly well within the limits of error of the methods, but it may none the less represent a real difference, since traces of other α-keto acids are present in blood.

With the present method a faint spot (R_F about 0·75), which is not given by standard solutions, is constantly obtained from blood, cerebrospinal fluid and urine. In blood, the substance giving this spot seems to be present entirely or almost entirely in plasma. One other faint spot (R_F about 0·83) has been rarely observed. These two spots probably correspond with the similar faint spots described by Kulonen, Carpén & Ruokolainen (1952) and by Gey (1954).

Acetoacetate has been detected occasionally in blood taken from non-diabetic subjects in the post-absorptive state, but only in very small amounts. Its presence may have occasionally contributed to the slightly higher blood α-keto acid levels found by the Friedemann & Haugen method, but it is more probable that the unidentified hydrazone of R_F 0·75 is responsible, since it is present in amounts which, on elution, give a colour intensity comparable with that given by α-oxoglutarate.

Table 1. *Analysis of solutions containing pure pyruvic and α-oxoglutaric acids*

No. of determinations	Present (μg.)	Found (μg.) Mean	Range	S.D.
		Pyruvate		
15	24	23·9	22·2–26·5	1·22
15	40	39·6	37·1–40·7	1·33
5	60	62·5	60·7–63·5	—
		α-Oxoglutarate		
15	20	19·9	18·7–22·0	0·58
15	40	40·1	38·2–42·1	1·07
5	60	60·1	59·1–61·2	—

Table 2. *Recoveries of 30 μg. amounts of pure pyruvic or α-oxoglutaric acids added to 16 ml. amounts of metaphosphoric acid filtrates of blood or of diluted urine*

	No. of determinations	Content before additions (μg.)	Recovery Mean	S.D.
		Blood filtrates		
Pyruvate	12	14·9–20·8	93·8	5·7
α-Oxoglutarate	12	3·9–4·2	98·6	4·1
		Urines		
Pyruvate	9	16·8–21·5	93·2	4·2
α-Oxoglutarate	9	24·8–30·2	109·6	6·5

Table 3. *Comparison between the α-keto acid content of 100 consecutive samples of human blood as determined by the Friedemann & Haugen method and the combined pyruvate and α-oxoglutarate content estimated chromatographically*

Results are expressed as mg. of pyruvate/100 g.

	Mean	Range	S.D.
A. Friedemann & Haugen method	1·01	0·42–3·31	0·528
B. Chromatographic method	0·97	0·33–3·34	0·542
C. Differences *B* – *A*	−0·04	+0·27 to −0·35	0·102

Table 4. *Pyruvate and α-oxoglutarate contents of blood from resting healthy subjects in the post-absorptive state, and of cerebrospinal fluid from patients with neurological disease*

The number of subjects is given in parentheses

	Pyruvate (mg./100 g.)			α-Oxoglutarate (mg./100 g.)	
	Mean	Range	S.D.	Mean	Range
Blood (8)	0·56	0·36–0·81	—	0·12	0·08–0·18
Cerebrospinal fluid (71)	0·96	0·49–1·69	0·25	<0·02	tr.–0·06

Table 5. *Excretion rates of pyruvate and α-oxoglutarate in the urine of healthy subjects*

Collection period	Subjects		Pyruvate (mg./hr.)			α-Oxoglutarate (mg./hr.)		
	No.	Sex	Mean	Range	S.D.	Mean	Range	S.D.
Overnight	25	M.	0·37	0·14–0·64	0·09	0·92	0·27–1·70	0·38
Overnight	25	F.	0·33	0·23–0·50	0·07	1·60	0·68–3·18	0·55
24 hr.	6	M.	0·42	0·24–0·87	—	1·05	0·45–1·46	—

The values given in Table 3 are for blood obtained under diverse circumstances, and can only be compared therefore with other published values for human blood in a general way. To allow a more exact comparison, results are given in Table 4 for post-absorptive human blood taken from healthy subjects who had been lying on a couch for 30 min. or more. The sum of the mean levels of pyruvate and α-oxoglutarate (expressed as pyruvate) are lower than in the larger series. Values for these acids were also estimated in the cerebrospinal fluid of seventy-one patients with neurological disorders (Table 4). The striking feature is the low level of α-oxoglutarate in the cerebrospinal fluid as compared with that of the blood.

In contrast to blood and cerebrospinal fluid, the α-oxoglutarate content of urine is usually greater than that of pyruvate. In Table 5 the hourly nocturnal excretion rates for these acids have been given; the overnight urine (formed during 7–9 hr. of sleep) was chosen to exclude as far as possible the effects of varying amounts of exercise and of diet. It can be seen that a woman, despite her smaller size, excretes almost twice as much α-oxoglutarate in her urine as a man. To facilitate comparison with other published figures, the results are given of the mean hourly excretion rates of six healthy men over 24 hr. In these six men the mean excretion of pyruvate was 56 %, and of α-oxoglutarate 29 %, higher during the day than at night.

DISCUSSION

The results obtained show that it is possible with this simplified procedure to estimate the levels of pyruvate and α-oxoglutarate in biological fluids with reasonable accuracy. It is true that there is an appreciable loss, but the loss is constant and some of it due to the increase in volume of NH_3 soln. used for the extraction of the hydrazones from the ethyl acetate. Incomplete extraction of the hydrazones by the small volume of NH_3 soln. is a further source of loss, but it is probable that, as with the sodium carbonate extraction in the Friedemann & Haugen method, the proportion extracted remains constant. It seems likely also that the greater loss of pyruvate as compared with α-oxoglutarate is due to the conversion of a proportion of the pyruvate hydrazone into the isomer with R_F 0·67, since the colour equivalent of the more rapidly moving isomer after the addition of the NaOH–Na_2CO_3 mixture is considerably less than that of the other isomer (Isherwood & Cruickshank, 1954; Markees, 1954, 1955). The relative proportion of the isomer of R_F 0·67 tends to be rather variable, but in the last fifty estimations on blood the proportion present has only been 12·5 % (S.D. 5·15).

The values for pyruvate and α-oxoglutarate in human blood correspond fairly closely with the mean levels of pyruvate (0·35–0·92 mg./100 ml.) and of α-oxoglutarate (0·095–0·16 mg./100 ml.) found by other workers who have used chromatographic methods (Seligson, McCormick & Soborov, 1952; El Hawary & Thompson, 1953; Henneman, Altschule, Goncz & Alexander, 1954; Smith & Taylor, 1955). The α-keto acids do not appear to have been examined chromatographically in the cerebrospinal fluid before, but since pyruvate is the only one present in significant amounts, it is possible to make a reliable comparison with results obtained by colorimetric methods. The mean level of 0·96 mg./100 ml. found in the present series is in good agreement with comparable groups of patients reported by Bueding, Wortis & Stein (1942), and Amatuzio & Nesbitt (1950).

Chromatographic separation of the different α-keto acids is particularly necessary in urine, since in human urine α-oxoglutarate tends to be present

in greater amount than pyruvate, and other α-keto acids are also present in small amounts. Kulonen *et al.* (1952) reported a mean daily excretion in five subjects of 16·9 mg. of pyruvate and 14·0 mg. of α-oxoglutarate. The level of activity of the subjects is not mentioned, but it seems likely that the high pyruvate excretion may have been the result of exercise, since it has been shown by Johnson & Edwards (1937) and by McArdle (unpublished work) that the excretion of pyruvate is greatly increased after strenuous exertion. A point of some interest that is being further investigated is the higher excretion rate of α-oxoglutarate in women.

SUMMARY

1. A simplified procedure is described for the separation of the 2:4-dinitrophenylhydrazones of α-keto acids by paper chromatography, and for the quantitative estimation of the separated hydrazones of pyruvate and α-oxoglutarate.

2. Values are given for pyruvate and α-oxoglutarate in post-absorptive blood from healthy resting human subjects, in cerebrospinal fluid and in urine.

3. The rate of excretion of α-oxoglutarate in urine is greater in women than in men.

This work was carried out on behalf of the Medical Research Council. I wish to thank Professor R. H. S. Thompson and Dr M. F. S. El Hawary for help and advice. I am greatly indebted to Miss H. Pels and Miss F. Crew for their skilled assistance.

REFERENCES

Amatuzio, D. S. & Nesbitt, S. (1950). *J. clin. Invest.* **29**, 1486.
Bueding, E., Wortis, H. & Stein, M. (1942). *J. clin. Invest.* **21**, 85.
Cavallini, D., Frontali, N. & Toschi, G. (1949*a*). *Nature, Lond.*, **163**, 568.
Cavallini, D., Frontali, N. & Toschi, G. (1949*b*). *Nature, Lond.*, **164**, 792.
El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Gey, K. F. (1954). *Hoppe-Seyl. Z.* **294**, 128.
Henneman, D. H., Altschule, M. D., Goncz, R. M. & Alexander, L. (1954). *Arch. Neurol. Psychiat., Chicago*, **72**, 688.
Isherwood, F. A. & Cruickshank, D. H. (1954). *Nature, Lond.*, **173**, 121.
Johnson, R. E. & Edwards, H. T. (1937). *J. biol. Chem.* **118**, 427.
Kulonen, E., Carpén, E. & Ruokolainen, T. (1952). *Scand. J. clin. Lab. Invest.* **4**, 189.
Markees, S. (1954). *Biochem. J.* **56**, 703.
Markees, S. (1955). *Experientia*, **11**, 205.
Seligson, D., McCormick, G. J. & Soborov, V. (1952). *J. clin. Invest.* **31**, 661.
Smith, M. J. H. & Taylor, K. W. (1955). *Lancet*, **1**, 27.

Studies on the Particulate Components of Rat Mammary Gland

1. A METHOD FOR DETERMINING THE COMPOSITION OF THE RETAINED FLUID

By A. L. GREENBAUM and T. F. SLATER
Department of Biochemistry, University College, London

(*Received* 15 *November* 1956)

The mammary gland of the rat as dissected from the animal is a complex system containing not only mammary tissue itself but also the secreted fluid retained in the alveoli and duct system of the gland (colostrum in pregnancy, milk during lactation). Tissue suspensions of rat mammary gland prepared at different stages of the lactation cycle contain a variable quantity of this retained fluid which, in the later stages of lactation, may account for up to 60% of the gross weight of the gland (Folley & Greenbaum, 1947). As the retained fluid has neither the same chemical constitution nor the same enzymic activity as the parent mammary tissue it is essential, in quantitative studies on mammary tissue, to correct analyses on the total gland for the amount of retained fluid found in the wet tissue when it is excised from the body.

A method has been published for the estimation of retained milk in suspensions of rat mammary tissue (Folley & Greenbaum, 1947) in which the percentage of lactose in milk obtained from lactating rats was determined and then used to calculate the percentage milk retained in tissue suspensions at all stages of the lactation cycle. While this method gave reasonable values for milk retention during lactation, where the percentage of lactose in milk was shown to be approximately constant, it was almost certainly in error when applied to rats at other stages of the lactation cycle such as late pregnancy, shortly after parturition or early in

involution. The percentage of lactose in the secreted fluid is different in late pregnancy compared with lactation. Colostrum has, in general, a lower lactose content than milk of the same species (Woodman & Hammond, 1923; Davies, 1936). The method proposed by Folley & Greenbaum (1947) is limited to periods where samples of retained fluid can be obtained for lactose analysis and so is not applicable to periods other than lactation. The present paper describes an indirect method for determining the lactose content of secreted fluid at certain stages when direct analysis is not possible.

In any mammary-tissue suspension, it is obvious that as the volume of retained fluid/gram increases so the tissue content/gram must decrease by a corresponding amount. If the amount of retained fluid and the tissue content could both be measured without error, the relation between the two measurements would be linear over the entire ranges 0–100 % (retained fluid) and 100–0 % (tissue). Since these quantities cannot, however, be measured directly it is necessary to estimate them indirectly. For any one stage in the lactation cycle the lactose content of the retained fluid is assumed to be constant, and measurements of lactose should therefore be proportional to the amount of retained fluid. Similarly, if some quantity proportional to the tissue content can be measured, the relation between this quantity and the lactose content would be linear.

It is not generally possible to obtain a direct estimate of the tissue present in mammary-gland suspensions in terms of nitrogen. The total nitrogen of the suspension contains a contribution resulting from the presence of nitrogen-containing compounds (casein, albumin, etc.) in the retained fluid; this contribution cannot be determined except in lactation. Some constituent, other than nitrogen, which is proportional to the tissue present must be used to obtain the correlation between tissue and lactose.

In the absence of mitosis, deoxyribonucleic acid (DNA) has been shown to be constant for each type of cell in a great variety of tissues (see Vendrely, 1955). In the absence of marked alterations in polyploidy or of multinucleate cell numbers, the DNA content of a mitotically quiescent tissue is directly proportional to the number of cells in that tissue. No alterations in the numbers of polyploid or multinucleate cells have been reported in the rat mammary gland. Sternberg (1950) has shown that there is no significant amount of either ribonucleic acid (RNA) or DNA in rat milk. Finally it will be shown in the second paper in this series (Greenbaum & Slater, 1957*a*) that, although a burst of mitotic activity occurs in the mammary gland some 30 hr. *post partum*, the period during which cell division occurs is sharply defined and is completed in a relatively short period; 24 hr. before, or after, this period of mitotic activity the tissue is mitotically quiescent. In a group of animals of the same functional status, the DNA contents of mitotically quiescent tissue samples are proportional to the tissue contents of the samples (see De Robertis, Nowinski & Saez, 1955). In more precise terms, Hoffman (1953) states that the two major parameters governing the frequency distribution of cell size in a tissue sample are the ages of the cells and the mitotic rate. Thus, in a group of animals with identical histories, and where mitotic activity is low, the frequency distributions of cell size are practically identical, so that in a large sample of tissue the tissue present is proportional to the number of cells present and hence to the DNA content of the sample.

These considerations apply to the mammary gland during the lactation cycle except at three periods: (*a*) during early pregnancy when rapid growth is occurring, (*b*) some 30 hr. after parturition when, for a brief period of some 12 hr., a burst of mitosis occurs, and (*c*) possibly during involution. It is therefore possible to use the level of DNA in a sample as a measure of the amount of tissue present in that sample except during the periods mentioned above. With DNA as a measure of the amount of tissue present, it is possible to use the relationship described above and hence to derive the percentage of lactose in the retained fluid. This figure, in turn, permits the calculation of the percentage of retained fluid in a tissue suspension. The indirect method described here is applicable to the estimation of retained fluid in the gland at the physiologically important times of late pregnancy, at parturition and during most of lactation.

METHODS

Definitions. (*a*) The term 'retained fluid' refers to the secretion which is retained in the ducts and alveoli of the excised gland (colostrum in pregnancy, and milk in lactation). (*b*) The term 'total nitrogen' refers to the nitrogen content of the whole mammary-tissue suspension. This includes the nitrogen content of the mammary tissue itself and also the nitrogen of the retained fluid. (*c*) The term 'tissue nitrogen' refers to the nitrogen contribution to the mammary suspension from the mammary tissue alone.

Animals. Adult, female albino rats of approximately 200 g. were used throughout this investigation. With rats needed for the 20-day pregnancy values, male rats were introduced into a cage of virgin females for one night only; the length of the ensuing pregnancy was measured from the day of removal of the males.

Rats were killed by dislocation of the cervical vertebrae. The abdominal glands were quickly dissected from the skin and placed in ice-cold 0·25 M-sucrose. After weighing, the glandular tissue was finely chopped with scissors, homogenized in a loose-fitting all-glass Potter–Elvehjem homogenizer (Potter & Elvehjem, 1936), and then re-homogenized

in a tight-fitting homogenizer having a polythene piston with a stainless-steel shaft. The suspension was diluted with cold 0·25 M-sucrose to give a 1 : 10 tissue suspension and then strained through muslin to remove intractable connective and fatty tissue. The above procedure, finally adopted after trying several variations such as a stainless-steel Potter–Elvehjem homogenizer and a Folley & Watson (1948) top-drive blendor, is very similar to that developed by Popják & Tietz (1954).

Estimation of lactose in mammary tissue. The method developed by Folley & Greenbaum (1947) for the estimation of lactose in mammary suspensions is satisfactory and has been used in this investigation.

Estimation of DNA in mammary-tissue suspensions. The DNA content of mammary-tissue suspensions was determined throughout this investigation by the diphenylamine reaction of Dische (1955). The procedure adopted was to extract the nucleic acids from 2 ml. of suspension by the procedure of Schneider (1945), and then to heat 2 ml. of extract with 4 ml. of diphenylamine reagent (Dische, 1955) for 15 min. on a boiling-water bath. After cooling, the colour was measured on a Unicam SP. 500 spectrophotometer at 600 mμ. In order to make the values comparable with other figures in the literature the DNA values have been calculated as deoxyribonucleic acid phosphorus (DNA-P).

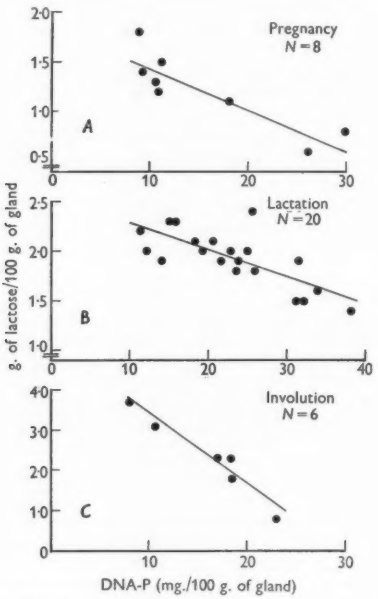


Fig. 1. Calculation of the lactose content of rat milk. *A*, At the 20th day of pregnancy; *B*, figures taken for all stages of lactation; *C*, on the 2nd day of mammary involution. The lactose content is calculated (as g./100 g. of milk) from the equation of the regression line.

RESULTS

The results of the determinations of lactose and DNA have been grouped into sets of distinct functional status and are given in Table 1. The regression equation relating the lactose content with the DNA-P content of the mammary suspensions are also included.

Fig. 1 shows the individual lactose and DNA-P values used in calculating the regression equations given in Table 1. The percentage of lactose in the retained fluid for each stage of the lactation cycle investigated was derived from the equation of the regression line as mentioned above. It was not possible to separate regressions corresponding to the third, tenth or eighteenth day of lactation so that a constant lactose content in milk throughout lactation is indicated by this method. The values for the percentages of lactose in retained fluid were then coupled with experimentally determined lactose contents for each mammary suspension to calculate

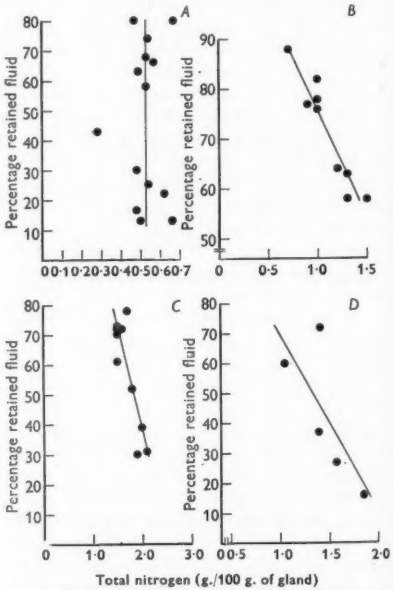


Fig. 2. Calculation of the nitrogen content of rat milk and mammary tissue. *A*, At the 20th day of pregnancy; *B*, at the 3rd day of lactation; *C*, at the 18th day of lactation; *D*, on the 2nd day of mammary involution. The nitrogen content of milk (g./100 g. of milk) is obtained from the regression line as the abscissa corresponding to 100% retained fluid on the ordinate. The nitrogen of the mammary tissue (g./100 g. of milk-free tissue) is obtained from the regression line as the abscissa corresponding to 0% retained fluid on the ordinate.

Table 1. *Relationship between lactose and DNA-P: the lactose content of retained fluid, and the level of retained fluid in rat mammary gland*

Status	DNA-P: No. of animals	DNA-P: mg./100 g. wet wt. of gland	Regression of lactose content on DNA-P content: No. of animals	Equation	r	Lactose content of retained fluid (g./100 g. of fluid)	Lactose g./100 g. wet wt. of gland (range)	Mean percentage of retained fluid in fresh gland
Virgin	6*	4·8±0·1†	—	—	—	—	—	—
Pregnant (20 days)	11	14·7±2·2	8	Lactose = 1·85–0·041 (DNA-P)	−0·85	1·85	1·21 (0·60–1·80)	66
Lactating (3 days)	10	22·9±2·3					1·95 (1·52–2·33)	76
Lactating (10 days)	6	20·7±1·9	20	Lactose = 2·55–0·027 (DNA-P)	−0·73	2·55	1·98 (1·85–2·09)	77
Lactating (18 days)	9	25·2±2·6					1·88 (1·39–2·39)	72
Involuting (2 days)	9	17·5±3·1	6	Lactose = 5·11–0·175 (DNA-P)	−0·97	5·11	2·35 (0·80–3·70)	46

* Analyses on two pooled samples; three animals were used per sample.
† In all tables the values given are group means±standard error of the mean.

Table 2. *Relationship between the level of retained fluid and total nitrogen: tissue protein, and the protein of the retained fluid in rat mammary gland*

Status	Total nitrogen: No. of animals	Total nitrogen: g./100 g. wet wt. of gland	Regression of the percentage of retained fluid (R.F.) on the total nitrogen content of mammary-tissue suspensions: No. of animals	Equation	r	Tissue protein*: g./100 g. wet wt. of gland	Tissue protein*: g./total gland	Protein content of retained fluid (g./100 g. of fluid)
Virgin	6†	0·266±0·020	—	—	—	1·70	0·043	—
Pregnant (20 days)	10	0·504±0·037	14	% R.F. = 16 467 (Total N) − 8612	+0·016	1·17	0·054	3·4
Lactating (3 days)	9	1·083±0·085	9	% R.F. = 118·4 − 42·5 (Total N)	−0·95	4·28	0·193	2·8
Lactating (10 days)	6	0·921±0·076	—	—	—	3·91‡	0·238‡	—
Lactating (18 days)	8	1·513±0·050	10	% R.F. = 177·4 − 69·96 (Total N)	−0·87	4·54	0·254	7·1
Involuting (2 days)	6	1·207±0·117	5	% R.F. = 129·3 − 59·95 (Total N)	−0·75	7·45	0·491	3·1

* Calculated from the mean percentage of retained fluid (R.F.) in Table 1.
† Analyses on two pooled samples; three animals were used per sample.
‡ Based on a protein content of milk-free gland equal to the average of the third and eighteenth day lactation groups.

the percentage of retained fluid in each suspension. The percentage of lactose in the retained fluid at each stage of the lactation cycle, as derived from the regression lines, and the mean percentages of retained fluid in the groups of mammary suspensions are given in Table 1.

Determination of the contributions by tissue nitrogen and retained-fluid nitrogen to the total nitrogen of mammary-gland suspensions

The total nitrogen content of suspensions of rat mammary gland at the six stages of the lactation cycle considered are given as mean values in Table 2.

The total nitrogen of the suspension is the sum of two contributions: the nitrogen content of the gland tissue and the nitrogen content of the retained fluid present. For the stages of late pregnancy, early lactation and involution, the same difficulties as those affecting direct lactose analysis operate to prevent direct analysis of the nitrogen content of the retained fluid. However, the basic assumption used in the lactose intercept procedure can be used to overcome the difficulties at the periods studied. If the same assumption is made with respect to the nitrogen of the gland as was made for the lactose, i.e. that at each particular stage of the lactation cycle both the retained fluid and the tissue have a characteristic composition, then the following equation can be derived.

If R_i represents the fraction of fluid retained in the gland, T_i the total protein in the mammary-gland suspension, t the percentage of protein in mammary tissue, m the percentage of protein in retained fluid, then the identity:

total protein of the gland = tissue protein + retained fluid protein

can be written in the form

$$T_i = (1 - R_i)\, t + R_i m,$$

so that

$$T_i = R_i(m - t) + t.$$

If the basic assumption that t and m are characteristic constants is valid, a graph plotting T_i against R_i will be linear. The constant t will give the percentage of protein in mammary tissue and the value of T_i when R_i equals 1 is identical with the percentage of protein in retained fluid.

The individual determinations of the total nitrogen of the mammary-gland suspensions are plotted against the calculated percentage of milk retention in Fig. 2 for the stages investigated. The calculated regression equations of the percentage retained fluid on total nitrogen are given in Table 2, which also includes the values calculated for the percentage protein content in tissue and retained fluid. No regression line could be obtained with the tenth day lactating group, where the retained fluid contents were too closely grouped to obtain a reliable estimate.

DISCUSSION

The method proposed here for the indirect analysis of retained fluid at periods of the lactation cycle where direct analysis is not possible is based on several basic assumptions whose validity will now be considered.

First, it is assumed that at each stage of the lactation cycle studied a characteristic composition exists both for mammary tissue and for retained fluid, i.e. that there would be little difference in composition and properties between individuals of each group of animals studied. This assumption seems reasonable, as none of the periods studied involved periods of rapid growth or very rapid changes in synthetic activity. Some direct evidence can, however, be derived from an examination of the individual standard errors of the means for various components of the mammary gland given in the next two papers (Greenbaum & Slater, 1957*a*, *b*). The small standard errors of the means of every component examined, at each stage of the lactation cycle investigated, indicates that members of each group do not depart significantly from a mean value identifiable as a group constant.

The second assumption was that, at the periods studied, the DNA-P content of a mammary-tissue suspension was directly related to the mammary tissue present. This assumption has been tested for the lactation period. Samples of rat milk, obtained after the injection of oxytocin, were analysed for total protein and lactose. From these values for milk composition, and the lactose and total nitrogen contents of the mammary suspensions from lactating rats, it was possible to calculate the contributions to the total-suspension nitrogen from the mammary-tissue nitrogen and the retained-fluid nitrogen respectively. The assumption that tissue nitrogen (i.e. tissue protein) is directly related to the DNA-P content of the sample can then be tested by plotting the tissue-nitrogen values against the DNA-P contents. Fig. 3 shows the relationship between DNA-P and tissue nitrogen obtained in this way. From the variability of milk composition reported by Cox & Mueller (1937) it can be seen that the assumption relating DNA-P to tissue nitrogen appears justified at least for the lactation period. It is not possible to verify the assumption for the periods of pregnancy and mammary involution as adequate samples of retained fluid for direct analysis cannot be obtained. However, the verification of the assumption in lactation makes its application to other stages of the lactation cycle not unreasonable, particularly as mitotic activity is rarely found in any of the stages considered.

The basic argument that DNA-P and lactose are linearly related is a corrollary of the two preceding assumptions. Independently the argument seems

justified by consideration of the graphs shown in Fig. 1*A–C*. The linearity of the data in the graphs was tested by fitting both a straight line and a quadratic curve to the data. In no case was the variance about the line significantly greater than that about the curve, indicating that the data were not inconsistent with linearity.

From the figures obtained by the direct analysis of milk samples collected during lactation, it is possible to check the values derived by the 'intercept procedure' for the lactation groups. Table 3 shows a comparison between the values obtained by direct analysis of rat milk for lactose and total protein and the corresponding values calculated by the intercept procedure. The protein values refer to the group at the eighteenth day of lactation. The values found by direct analysis differ from those given by Cox & Mueller (1937) and Folley & Greenbaum (1947), but the difference is probably due to the different strain and diet used, both of which have a considerable effect on milk composition (Cox & Mueller, 1937). It can be seen from Table 3 that the interecept method and direct analysis give closely similar results both for lactose and total protein. The application of the intercept method to the other periods of the lactation cycle studied, where direct analysis is not possible, therefore seems justifiable.

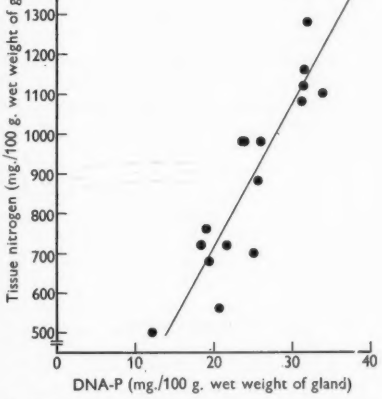


Fig. 3. Relation between mammary-tissue nitrogen and DNA-P.

Milk lactose and protein

The results given in Table 1 show that the lactose content of colostrum is smaller than for milk, and that the lactose content of the retained fluid increases in early involution. The rise in the lactose content of the retained fluid over parturition is in agreement with previous work on all other species examined (Davies, 1936).

The rat appears to differ from the larger mammals, however, in the changes in protein content of the retained fluid over the period of parturition. Whereas the general picture is that of a high level of protein (mostly albumins and globulins) in colostrum and a much lower level of protein (mostly casein) in milk, the rat appears to show only a slight fall in the protein content of the retained fluid over parturition, but an increase in the protein content of milk from the third to the eighteenth day of lactation (Table 2). From the data available it is not possible to decide whether the increase in protein content is a rapid one levelling out to a plateau early in lactation, or is the result of a sustained rise throughout the lactation period. The necessity for correcting analyses on rat mammary tissue for the presence of retained fluid has already been stressed.

The question arises (i) whether to use the experimentally found values for the lactose content of retained fluid where this is possible (i.e. in lactation) and the indirectly calculated lactose values for other groups (i.e. pregnancy and involution); or (ii) to use indirectly calculated values for all groups considered. Although experimentally determined values are to be preferred to calculated values, the small difference between the experimental and calculated figures for the lactation period (Table 3) is not sufficient to outweigh the advantage gained by better comparisons between groups when the results have all been calculated by the same procedure. We have therefore used calculated values for the percentage of lactose in retained fluid to

Table 3. *Direct analysis of milk, and values derived by the 'intercept procedure' for rats in lactation*

	Lactose* (g./100 g. of milk)			Protein† (g./100 g. of milk)		
	No. of animals	Mean	Standard error of estimate	No. of animals	Mean	Standard error of estimate
Intercept-method	20	2·55	0·14	10	7·1	1·5
Direct analysis	7	2·45	0·30	4	7·6	0·5

* Average for animals taken at various stages throughout lactation.
† Average for animals at the eighteenth day of lactation only.

convert the experimentally determined lactose of the whole homogenate into percentages of retained milk. We believe that, although the absolute magnitudes of the percentages of the retained fluid obtained may be slightly in error, the comparisons between groups of animals will be closer to reality.

Tissue protein

The rapid rise in tissue protein at parturition is of particular interest. The results obtained indicate that the protein content of the retained fluid is sensibly the same for the late-pregnancy and early-lactation groups (Table 2). Since the percentage of retained fluid in the glands does not appear to alter markedly over parturition (Table 1) the rapid increase in total protein can be taken to indicate an increase in cellular material. This is further indicated by the results for tissue protein (Table 2). The rapid increase in tissue protein over parturition can hardly be the result of a cellular hypertrophy, as the available cytological evidence suggests that mammary cells are smaller in early lactation than in late pregnancy (Weatherford, 1929; Jeffers, 1935). The more likely explanation is that an extensive cell division occurs at parturition. Evidence is presented in Part 2 (Greenbaum & Slater, 1957*a*) that such a mitotic wave does, in fact, occur.

The rapid rise in tissue protein over parturition is followed by a small increase in tissue protein throughout lactation when calculated both as protein/gland and as protein/100 g. of wet tissue. The rise in tissue protein/gland during pregnancy is surprisingly small, but is probably accounted for by the fact that the rats used in this investigation were fully adult females in which the virgin glands had been built up by passage through several oestrous cycles (Kirkham & Turner, 1953). The results for tissue protein in lactation are consistent with the cytological findings of Weatherford (1929) that mitosis is virtually absent from lactating glands although the mammary cells show a progressive hypertrophy. Such a hypertrophy could be the consequence of an increasing water content, a common feature both of synthetically active and rapidly growing cells (see Cowdry, 1955). Although Smith (1956) found no change in the water content of the guinea-pig mammary gland, it should be remembered that the lactation curve of the guinea pig is very different from that of the rat, and Smith's observation does not exclude the possibility of hydration of the rat gland. Indeed, such hydration has in fact been described by Kleiber, Smith & Levy (1943) and by Folley & French (1949*b*) in rats. Another contribution to the hypertrophy in lactation could be due to an increase in the volume of cellular inclusions, particularly fat. Thus the hypertrophy observed cytologically could be due to either of the above causes as well as to a genuine cellular expansion concomitant with an overall increase in cytoplasmic material.

The mammary gland undergoes a period of intense chemical and physiological change at and near the time of parturition. In the course of a few hours the entire metabolic pattern of the gland is refashioned. Repeated investigations of the diverse features of the increased glandular activity, illustrated by the rise in R.Q. and Q_{O_2} (Folley & French, 1949*a*, *b*), the increase in enzymic activity (Folley & Greenbaum, 1947; Moore & Nelson, 1952; Greenbaum & Greenwood, 1954; Glock & McLean, 1954; Tuba, Orr & Wiberg, 1955), or in the total nitrogen and nucleic acids (Kirkham & Turner, 1953), have led to an appreciation of the magnitude, as well as the rapidity, of these changing patterns at this critical time. The results of this investigation have further illustrated the importance of this period.

SUMMARY

1. The levels of deoxyribonucleic acid, lactose and total nitrogen in suspensions of rat mammary-gland tissue have been determined at intervals during the lactation cycle.

2. A method is described by which the lactose and nitrogen levels of colostrum and milk at periods close to parturition can be determined.

3. Values have been calculated for the contribution by tissue and retained fluid to the total nitrogen of the suspensions. The tissue nitrogen increases slowly during pregnancy, very rapidly over the period of parturition and then slowly again during lactation.

We wish to acknowledge the advice and help of Mr N. W. Please of the Statistics department for assistance in the statistical aspects of this paper. Our thanks are also due to the Agricultural Research Council for an expenses grant to one of us (A.L.G.).

REFERENCES

Cowdry, E. V. (1955). In *Cancer Cells*. Philadelphia: W. B. Saunders and Co.

Cox, W. M. jun. & Mueller, A. J. (1937). *J. Nutr.* **13**, 249.

Davies, W. L. (1936). In *The Chemistry of Milk*. London: Chapman and Hall Ltd.

De Robertis, E. D. P., Nowinski, W. W. & Saez, F. A. (1955). In *General Cytology*, p. 68. Philadelphia: W. B. Saunders and Co.

Dische, Z. (1955). In *The Nucleic Acids*. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Folley, S. J. & French, T. H. (1949*a*). *Biochem. J.* **45**, 117.

Folley, S. J. & French, T. H. (1949*b*). *Biochem. J.* **45**, 270.

Folley, S. J. & Greenbaum, A. L. (1947). *Biochem. J.* **41**, 261.

Folley, S. J. & Watson, S. C. (1948). *Biochem. J.* **42**, 204.

Glock, G. E. & McLean, P. (1954). *Biochem. J.* **56**, 171.

Greenbaum, A. L. & Greenwood, F. C. (1954). *Biochem. J.* **56**, 625.
Greenbaum, A. L. & Slater, T. F. (1957*a*). *Biochem. J.* **66**, 155.
Greenbaum, A. L. & Slater, T. F. (1957*b*). *Biochem. J.* **66**, 161.
Hoffman, J. G. (1953). In *The Size and Growth of Tissue Cells*. Springfield, U.S.A.: Charles C. Thomas.
Jeffers, K. R. (1935). *Amer. J. Anat.* **56**, 257.
Kirkham, W. R. & Turner, C. W. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 123.
Kleiber, M., Smith, A. H. & Levy, P. (1943). *Proc. Soc. exp. Biol., N.Y.*, **53**, 94.
Moore, R. O. & Nelson, W. L. (1952). *Arch. Biochem. Biophys.* **36**, 178.
Popják, G. & Tietz, A. (1954). *Biochem. J.* **56**, 46.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
Smith, T. C. (1956). *Arch. Biochem. Biophys.* **60**, 485.
Sternberg, M. J. (1950). *C.R. Soc. Biol., Paris*, **230**, 2341.
Tuba, J., Orr, P. F. & Wiberg, G. S. (1955). *Canad. J. Biochem. Physiol.* **33**, 904.
Vendrely, R. (1955). In *The Nucleic Acids*. Ed. by Chargaff, E. and Davidson, J. N. New York: Academic Press Inc.
Weatherford, H. L. (1929). *Amer. J. Anat.* **44**, 199.
Woodman, H. E. & Hammond, J. (1923). *J. agric. Sci.* **13**, 180.

Studies on the Particulate Components of Rat Mammary Gland

2. CHANGES IN THE LEVELS OF THE NUCLEIC ACIDS OF THE MAMMARY GLANDS OF RATS DURING PREGNANCY, LACTATION AND MAMMARY INVOLUTION

BY A. L. GREENBAUM AND T. F. SLATER
Department of Biochemistry, University College, London

(*Received* 15 *November* 1956)

The deoxyribonucleic acid (DNA) content of any particular type of cell appears to be constant (see Vendrely, 1955). This constancy of content has been repeatedly demonstrated and deviates only in cells displaying polyploidy, or in multinucleate varieties. Furthermore, isotope-turnover studies indicate that DNA, except in mitotically active cells, is metabolized to a very limited degree (Hevesy & Ottesen, 1943). DNA has, therefore, come to be used as a reference standard directly related to the number of cells present (see Davidson, 1953; Davidson & Leslie, 1950). The value of such a reference standard in studies of the mammary gland, where total nitrogen is not necessarily related to the amount of tissue present, is obvious.

Ribonucleic acid (RNA) is considered to be of importance in the synthesis of protein (see Brachet, 1955). Although the intracellular site of protein synthesis is not fully established, many workers believe it to be in the small-particle fractions of the cytoplasm, the microsomes and submicrosomes (see Brachet, 1955). RNA, however, is widely distributed in the cell and the rapid turnover rate of nuclear RNA (Smellie, McIndoe, Logan, Davidson & Dawson, 1953) indicates that the RNA of the nucleus is probably of considerable importance in protein synthesis.

The mammary gland at parturition undergoes a rapid and intense change in the rate of protein synthesis, lactation being well established within a few hours *post partum*. The amount of casein synthesized/day by an active rat mammary gland is comparable in amount with the parent tissue present. The mammary gland has, therefore, advantages to offer over other tissues, where the rate of protein synthesis is constant, in studies relating RNA to protein synthesis.

METHODS

Animals. Rats were killed by cervical dislocation and the entire lower abdominal mammary glands quickly removed and placed in ice-cold 0·25 M-sucrose.

Seven groups of female albino rats were used: (*a*) adult virgin rats aged 4–6 months; (*b*) adult rats, about 200 g. body wt. and 20 days pregnant; (*c*) adult rats 3, 10 and 18 days *post partum*; (*d*) adult rats in the second and fourth days of mammary involution. Litters, which in all cases were reduced to 6–8 pups, were weaned 22 days *post partum*.

Homogenizing. After homogenizing by the procedure outlined in the previous paper (Greenbaum & Slater, 1957*a*) the homogenate was diluted with 0·25 M-sucrose to give a 1:10 tissue suspension. Portions of this suspension were used for total-nitrogen determination, nucleic acid determinations and lactose estimation to determine the milk content of the suspensions.

Centrifuging. The mammary gland suspension (30 ml.) was centrifuged to obtain a large-particle (mitochondrial) fraction. The procedure was as follows: after two initial centrifugings at 600 *g* for 10 min. in a refrigerated centrifuge to remove cellular debris and nuclei, the suspension was centrifuged at 8000 rev./min. for 15 min. at plateau speed in a Servall refrigerated centrifuge (Model SS 2). The centrifuge constants for this stage were (Duve & Berthet, 1953): $r_{(min.)} = 11$ cm.; $r_{(max.)} = 15$ cm.; $g_{(plateau)} = 10\,900$; temp., 0°. These values give a time integral of about 230 000 *g* min.

Separation of sediment and supernatant suspension was achieved by decantation. The fatty layer, which was solid at the temperature used in centrifuging, was removed in

the process of decanting. The mitochondrial fraction was resuspended in the original volume of ice-cold 0·25 M-sucrose.

Chemical methods

Total nitrogen. This was estimated by a micro-Kjeldahl method.

Lactose. This was determined in the suspensions of mammary gland by the method of Folley & Greenbaum (1947). The percentage of retained fluid in the mammary gland was calculated by the method of Greenbaum & Slater (1957*a*).

Extraction of nucleic acids. Nucleic acids were separated from the tissue fractions by the procedure of Schneider (1945) in preference to that of Schmidt & Thannhauser (1945). An objection to the Schmidt & Thannhauser procedure is that phosphorus-containing compounds other than nucleic acids come through the extraction procedure and give high ribonucleic acid phosphorus (RNA-P) values compared with those from pentose determinations on the same samples (see Davidson, 1953). Further, as both DNA and RNA are estimated by phosphorus content in the Schmidt & Thannhauser method, the hydrolysis stage is critical and the accuracy of the final estimations will be a reflexion of the resolution of this step.

Schneider (1945) has observed that in many tissues the phospholipid extraction may be omitted without affecting the nucleic acid estimation, and we have confirmed that this applies to the mammary gland. As a routine practice we have therefore left out the phospholipid extraction. The standard procedure adopted was to precipitate the nucleoproteins of 2 ml. of a 1:10 tissue suspension or of 2 ml. of a mitochondrial fraction with 2 ml. of 10% (w/v) trichloroacetic acid (TCA). After standing in ice for several hours the precipitate was centrifuged off, washed twice with 5% TCA, and then heated for 15 min. at 90° with 5 ml. of 5% TCA. The residue was centrifuged off and reheated with a further 5 ml. of 5% TCA for the same time. The combined supernatants were filtered and used for the estimation of nucleic acids by determination of the pentose or deoxypentose content, which were assumed to be related to the RNA and DNA content respectively.

DNA was measured by the method of Dische (1955) at 600 mμ on a Unicam SP. 500 spectrophotometer. RNA was measured by the modification of the method of Mejbaum (1939) described by Albaum & Umbreit (1947), with 45 min. as the heating time for colour development.

The RNA value was corrected for the interference due to the DNA present, which also gives a colour under the conditions of the RNA estimation. Interference from the sucrose of the suspending medium and from the lactose of the milk retained in the gland was found to be negligible after two washes with cold 5% TCA before the nucleic acid extraction. Results for both types of nucleic acid were calculated in terms of nucleic acid phosphorus, i.e. deoxyribonucleic acid phosphorus (DNA-P) and RNA-P (see Leslie, 1955).

RESULTS

Nitrogen distribution

The results of the determination of nitrogen in the whole gland and in the mitochondrial fraction are shown in Table 1. The total nitrogen, when expressed as mg. of N/100 g. wet wt. of gland, increases throughout pregnancy and lactation and falls abruptly in mammary involution. The rise in pregnancy represents a doubling of the nitrogen content of the virgin gland, and a further doubling occurs over the period of parturition to the third day of lactation. During lactation the nitrogen increases further, so that by the eighteenth day of lactation it is 1·5 times as high as at the third day of lactation. The values at 10 days appear to be anomalous in that they show a decrease in magnitude compared to the values at 3 days. Observations of similar anomalous values for rats at mid-lactation have been reported by Kirkham & Turner (1953). The fall in nitrogen content at 10 days is not observed if the total nitrogen content of the entire abdominal glands is considered (Table 1, column 6) due to the increased weight of the glands (Table 1, column 3).

Values for mitochondrial nitrogen, expressed as mg. of N/100 g. wet wt. of gland, also show the same type of rise throughout pregnancy and lactation, although in this case the rise during pregnancy (eightfold) is relatively much greater (double) than that found in whole-tissue nitrogen. At parturition there is a sharp rise in mitochondrial nitrogen, the value on the third day of lactation being double that found at the end of pregnancy. A similar pattern of increase up to the end of the lactation period, followed by a fall in the involution period, is shown when values for the mitochondrial nitrogen content of the entire gland are calculated.

Variations in the distribution of RNA-P and DNA-P

The results showing the changes in the RNA-P and DNA-P content of the whole tissue and of mitochondrial RNA-P over the secretory cycle are shown in Table 2. The pattern of change in the content of RNA-P is very similar to that found for the changes in nitrogen content, i.e. a general increase throughout pregnancy and lactation, followed by a sharp drop during involution. It should be noted that there is a very considerable rise of both tissue and mitochondrial RNA-P at parturition. It will be seen from Table 2, column 13, that, except at the third day of lactation, the mitochondrial RNA-P is a relatively constant fraction of the total tissue RNA-P (about 15–20%), and is within the range normally quoted for the RNA-P content of mitochondrial fractions (Hogeboom, Schneider & Palade, 1948; Schneider & Hogeboom, 1951).

The results of the determination of the DNA-P content of rat mammary gland are also shown in Table 2 (columns 3–5). The main point of interest in these results is the rapid rise in DNA-P concentration at parturition from 40·4 to 81·4 mg. of DNA-P/100 g. of milk-free gland (Table 2, column 4).

Table 1. *Distribution of nitrogen in rat mammary gland*

Status	Wt. of gland: No. of animals	Wt. of gland: (g.)	Total nitrogen: No. of animals	Total nitrogen: mg./100 g. wet wt. of gland	Total nitrogen: mg./total gland	Mitochondrial nitrogen: No. of animals	Mitochondrial nitrogen: mg./100 g. wet wt. of gland	Mitochondrial nitrogen: mg./total gland	$\frac{\text{Mitochondrial nitrogen*}}{\text{Total nitrogen}} \times 100$
Virgin	6†	2·6±0·3‡	6	266±20	6·8±1·6	6	8·2±1·2	0·19±0·02	3·3±0·7
Pregnant (20 days)	11	4·6±0·3	10	504±37	22·9±1·9	7	65·7±14·7	2·9±0·6	14·4±2·1
Lactating (3 days)	10	4·5±0·3	9	1083±85	48·1±3·7	6	139·2±20·8	6·8±1·3	16·2±1·9
Lactating (10 days)	6	6·1±0·4	6	921±76	57·7±8·9	6	213·0±21·6	13·4±2·4	23·1±1·2
Lactating (18 days)	10	5·6±0·3	8	1513±59	81·5±4·7	8	271·8±28·3	13·8±1·8	20·0±1·9
Involuting (2 days)	6	6·6±1·0	6	1207±117	78·4±18·6	3	183·0±1·0	12·0±1·3	19·3±0·8
Involuting (4 days)	4	3·2±0·4	4	661±68	22·2±4·2	4	157·0±18·0	5·3±1·2	23·4±0·9

* Units: $\frac{\text{mg./100 g. wet wt. of gland}}{\text{mg./100 g. wet wt. of gland}}$.

† Analyses on two pooled samples; three animals were used per sample.

‡ In all tables the values given are group means±standard error of the mean.

Table 2. *Distribution of nucleic acids in rat mammary gland*

Status	DNA-P in tissue suspension: No. of animals	DNA-P in tissue suspension: mg./100 g. wet wt. of gland	DNA-P in tissue suspension: mg./100 g. of gland (milk-free)	DNA-P in tissue suspension: mg./total gland	RNA-P in tissue suspension: No. of animals	RNA-P in tissue suspension: mg./100 g. wet wt. of gland	RNA-P in tissue suspension: mg./100 g. of gland (milk-free)	RNA-P in tissue suspension: mg./total gland
Virgin	6‡	4·8±0·1	—	0·12±0·04	6	2·7	—	0·06±0·01
Pregnant (20 days)	11	14·7±2·2	40·4±3·1	0·71±0·15	11	17·7±2·1	55·2±8·9	0·85±0·14
Lactating (3 days)	10	22·9±2·3	81·4±2·9	1·02±0·10	8	37·9±3·9	156·0±14·4	1·73±0·14
Lactating (10 days)	6	20·7±1·9	80·5±6·7	1·28±0·18	6	55·6±5·1	219·8±24·5	3·45±0·46
Lactating (18 days)	9	25·2±2·6	82·2±6·5	1·46±0·17	11	64·5±3·8	225·2±20·1	3·48±0·27
Involuting (2 days)	9	17·5±3·1	30·9±3·8	1·38±0·32	6	22·9±1·8	84·0±24·0	1·70±0·06
Involuting (4 days)	5	16·4±1·2	—	0·53±0·07	5	18·4±3·1	—	0·61±0·13

Status	RNA-P in mitochondrial suspension: No. of animals	RNA-P in mitochondrial suspension: mg./100 g. wet wt. of gland	RNA-P in mitochondrial suspension: mg./total gland	$\frac{\text{Mitochondrial RNA-P*}}{\text{Homogenate RNA-P}} \times 100$	$\frac{\text{Homogenate RNA-P†}}{\text{DNA-P}}$
Virgin	3	0·54	0·01	15	0·70
Pregnant (20 days)	9	3·8±0·9	0·17±0·05	20·1±2·9	1·22±0·15
Lactating (3 days)	7	9·9±1·2	0·49±0·06	27·9±4·3	1·86±0·13
Lactating (10 days)	6	11·8±2·8	0·77±0·23	16·7±2·0	2·72±0·19
Lactating (18 days)	9	11·9±1·1	0·64±0·06	21·1±2·2	2·69±0·23
Involuting (2 days)	4	4·1±1·1	0·32±0·11	18·4±5·7	1·63±0·43
Involuting (4 days)	4	4·1±1·5	0·15±0·06	21·7±4·8	1·08±0·13

* Units: $\frac{\text{mg./100 g. wet wt. of gland}}{\text{mg./100 g. wet wt. of gland}}$.

† Units: $\frac{\text{mg./total gland}}{\text{mg./total gland}}$.

‡ Analyses on two pooled samples; three animals were used per sample.

Throughout lactation the DNA-P concentration is remarkably constant, although the total DNA-P/gland increases slowly.

The ratio RNA-P:DNA-P is assumed to be a measure of RNA-P/cell, and this has been evaluated at the various stages considered (Table 2, column 14). It can be seen that the RNA-P content/cell increases during pregnancy and the first half of lactation, after which it levels off, finally falling during mammary involution.

Table 3 shows the variations with the secretory period of several other ratios, derived from the results listed in Tables 1 and 2.

The tissue-nitrogen values given were calculated by the intercept method previously published (Greenbaum & Slater, 1957*a*). The percentage of the tissue nitrogen which is recoverable as mitochondrial nitrogen is shown in column 3. As the tissue-nitrogen figure for each stage is a calculated mean value, no standard errors of the mean of ratios involving tissue nitrogen are available. The highest value for this ratio is found in the late pregnant gland and is about 35%, a figure which compares with values of about 25% found by other investigators for the nitrogen content of rat-liver mitochondria (see Schneider & Hogeboom, 1951). Since at all stages of the lactation cycle investigated here the mammary gland is a mitotically quiescent tissue, the DNA-P level is proportional to the number of cells present (Greenbaum & Slater, 1957*a*). The results for the mitochondrial nitrogen have therefore been related to amount per cell by considering the ratio of mitochondrial nitrogen to DNA-P (Table 3, column 4); and similarly the RNA-P content is compared to the mitochondrial nitrogen in columns 5 and 6.

Table 3. *Interrelationships between nucleic acid content and nitrogen content of rat mammary gland*

Status	Tissue nitrogen* (mg./total gland)	Mitochondrial N / Tissue N × 100†	Mitochondrial N‡ / DNA-P	Total RNA-P† / Mitochondrial N	Mitochondrial RNA-P† / Mitochondrial N
Virgin	6·8	2·8	1·7	0·42	0·060
Pregnant (20 days)	8·4	34·5	6·1±1·6	0·38±0·13	0·033±0·009
Lactating (3 days)	30·2	22·5	7·9±1·2	0·28±0·05	0·077±0·021
Lactating (10 days)	37·2	36·0	10·7±1·3	0·27±0·03	0·052±0·007
Lactating (18 days)	39·7	34·8	16·3±1·4	0·22±0·01	0·044±0·006
Involuting (2 days)	76·7	15·6	12·3±2·5	0·14±0·004	0·017±0·004
Involuting (4 days)	—	—	9·6±0·5	0·10±0·005	0·023±0·006

* From Greenbaum & Slater (1957*a*).

† Units: mg./total gland / mg./total gland.

‡ Units: mg./100 g. of fresh gland / mg./100 g. of fresh gland.

DISCUSSION

Ribonucleic acid

Two phases of protein synthesis may be recognized in the mammary gland. The first occurs during pregnancy and is associated with the growth of the gland itself; the second occurs after parturition, when protein synthesis is required not only for the maintenance of the existing mammary tissue but also for the elaboration of the protein constituents which are secreted in the milk. In view of current ideas linking RNA with protein synthesis it is of interest to consider variations in the level of RNA over the lactation cycle. The only previous work of this nature is, to our knowledge, that of Kirkham & Turner (1953), who described a gradual increase in the RNA content of the gland from pregnancy throughout lactation. They did not find the sharp increase in RNA content at parturition indicated by our results. The probable reason for this discrepancy is that Kirkham & Turner did not group

their animals but rather used animals scattered in all stages of the mammary cycle.

The results reported here are consistent with the idea of an RNA increase associated with the build up of the glandular material in early pregnancy and with the synthesis of colostrum proteins late in pregnancy. Comparison of the increases in the levels of tissue nitrogen, RNA-P and DNA-P indicates that the growth of the gland is hyperplastic and is in agreement with the many cytological studies of the gland during pregnancy (Roberts, 1921; Weatherford, 1929). There is a gradual increase in RNA throughout lactation, when expressed as RNA-P/100 g. wet tissue, concurrent with an increase in total nitrogen, but the DNA-P/100 g. wet tissue remains constant during this time. Thus the two main phases of protein synthesis are reflected by increases in the RNA-P. Although it is not possible to say whether the RNA is directly or indirectly related to protein synthesis, these results are further evidence for the corresponding times of appearance of both protein and RNA. It should be noted here that the transition at parturition is not from a tissue deficient in RNA-P to one rich in nucleic acid, but rather a transition from a tissue already rich to one extremely rich in RNA-P. Thus the values obtained from rats late in pregnancy and from those late in lactation were 55 and 225 mg. of RNA-P/100 g. wet wt. of gland (milk-free), respectively, compared with values of 72–99 mg. of RNA-P/100 g. of normal rat liver (Fukuda & Sibatani, 1953; Thomson, Heagy, Hutchison & Davidson, 1953) and of 198 mg. of RNA-P/100 g. of pancreas (Schneider, 1946).

Mitochondrial RNA

Mitochondrial RNA-P shows a similar pattern of change over the lactation cycle to that found for total RNA-P. The rapid increase in mitochondrial RNA-P at parturition, together with the similar increase in mitochondrial nitrogen (Tables 1 and 2) and succinic oxidase activity (Greenbaum & Slater, 1957*b*), lead to the view that the particles in the mitochondrial size range increase rapidly in numbers or size or both over this period. The conclusion of increased numbers has already been suggested by cytologists and confirmed by Howe, Richardson & Birbeck (1956), who found an increase in mitochondrial numbers per cell over parturition, but no significant change in the number of mitochondria per unit volume of cytoplasm.

The percentage of the total RNA-P found in the large-particle fraction isolated at 230 000 *g* min. is relatively constant at 15–20 % (Table 2, column 13). This is the same order as that found in rat-liver mitochondrial fractions, although the centrifugal field used here is much higher than is usual for the sedimentation of mitochondria.

The major part of the RNA-P of mammary tissue is in the particles smaller than the mitochondria: the mitochondria account for some 15–20 % of the total RNA-P and the nuclei for a further 10 % (Greenbaum & Slater, unpublished observations). Centrifuging a mammary-tissue suspension from lactating rats for $1{\cdot}5 \times 10^6$ *g* min. yields a supernatant which is virtually devoid of RNA-P, and it thus follows that some 70 % of the total RNA-P is to be found in those particles which fall in the microsomal and submicrosomal range. The changes in RNA-P content of the mammary gland at parturition seem to occur in these two fractions, a conclusion which is in agreement with other views linking protein synthesis and the microsomal fractions (see Brachet, 1955). The conclusion that the microsomal RNA-P/gland increases can be derived by subtracting the mitochondrial RNA-P/gland (Table 2, column 12) from the total RNA-P in the gland (Table 2, column 9), the difference being mainly the microsomal RNA-P/gland. The difference between these two columns increases from 0·05 to 0·68 mg. of RNA-P/gland during pregnancy, and to 2·84 mg./gland by the end of lactation.

Deoxyribonucleic acid

The increase in the DNA-P content of the gland found during pregnancy indicates a considerable degree of cellular proliferation. The DNA-P level at the end of pregnancy was six times that of the virgin gland, which, in the adult female rats used, already had some mammary development after its passage through several oestrous cycles.

The constancy of the DNA-P/100 g. wet wt. of gland (milk-free) (Table 2, column 4) throughout lactation appears to be in accord with such cytological evidence as is available and which claims that growth of the gland is complete by mid-pregnancy and that no mitotic figures are found in the gland after this time (Maeder, 1922; Weatherford, 1929). When, however, the constant DNA-P/100 g. wet wt. of gland (milk-free) found during lactation is considered in conjunction with the increase in total glandular DNA-P, a slow growth and expansion of the gland is indicated. The relatively slow nature of this increase in DNA-P/gland (1·4 times in the 15 days between the third and eighteenth days of lactation) might account for the failure of cytologists to observe cell division in the lactating gland. The sharp drop in DNA-P level in mammary involution is consistent with existing views of a breakdown of cellular material during this stage.

The period covering parturition requires special mention. Between the end of pregnancy and the third day of lactation the DNA-P level is doubled. It is almost certain that some of this increase may be accounted for by an increased leucocyte content

consequent upon a greater degree of vascularization, but this can hardly account for more than a small part of the rise. It seems more probable that the greater part of the rise is due to a hyperplasia of existing cellular tissue and, although this view is in conflict with older views, it has been confirmed by independent methods: (i) the observation that the number of cells per alveolus increases by some 70% from late pregnancy to early lactation (Jeffers, 1935); (ii) Lewin & Lewin (personal communication) have found a rise in the number of nuclei/mg. wet tissue of mouse mammary gland from 136 000 to 260 000 over the period from parturition to the second day of lactation. The increase in DNA-P reported here, and of nuclei found by Lewin & Lewin, suggests that a wave of mitosis occurs in the gland about the time of parturition. Such a wave could easily be completed in a few hours, and it is therefore not surprising that mitosis has not been observed in the few glands examined cytologically. Richardson, Slater & Greenbaum (unpublished results) have, however, made some preliminary observations (in which the mitoses were arrested by colchicine) which show that a wave of cell division occurs some 30 hr. after parturition and is of relatively short duration.

In the absence of significant mitosis, the ratio RNA-P:DNA-P is a measure of the RNA-P/cell. Since the stages of the lactation cycle studied in this investigation included neither the growth period of pregnancy nor the short period after parturition when mitosis occurs, the ratio can be used to follow the changes in the cellular content of RNA-P. It can be seen from Table 2 that there is a rapid rise in RNA-P/cell at parturition, but that the value increases only slowly as lactation advances. The ratio 2·72 for the mammary gland at the tenth day of lactation is surprisingly small for so active a tissue when compared with rat liver or pancreas, which have ratios of 4·38 and 4·1 respectively (Thomson *et al.* 1953; Schneider, 1946).

Mitochondrial nitrogen

The variations in mitochondrial nitrogen are shown in Table 1, and it now remains to correlate these changes with the changes in the tissue nitrogen of the mammary gland reported previously (Greenbaum & Slater, 1957*a*). A comparison of the tissue-nitrogen increase in pregnancy and lactation (Greenbaum & Slater, 1957*a*) with the increase in mitochondrial nitrogen reported here shows that in pregnancy and lactation the mitochondrial material builds up faster than the other fractions of the cell, whereas at parturition there is a more rapid increase of non-mitochondrial nitrogen.

The mitochondrial nitrogen/cell may be assessed by considering the ratio of mitochondrial nitrogen/DNA-P. This ratio (Table 3) rises throughout pregnancy and lactation, indicating a progressive rise in mitochondrial nitrogen/cell.

A further point of interest arises from columns 5 and 6 of Table 3. It can be seen that from late pregnancy to the end of lactation the ratio, whole-tissue suspension RNA-P:mitochondrial nitrogen, is sensibly constant; deviations from this constancy occur only in the virgin and involuting glands. However, the ratio mitochondrial RNA-P:mitochondrial nitrogen is by no means constant, indicating that the mitochondrial RNA-P and nitrogen are, to a certain extent, independent variables. The correspondence of total-suspension RNA-P and mitochondrial nitrogen leads, on the other hand, to the idea of a correspondence between mitochondrial material (represented by nitrogen) and the rate of protein synthesis (represented by the RNA-P of the whole suspension). It would be of particular interest in this connexion to study the changes in the microsomal and submicrosomal RNA-P throughout the lactation cycle.

The response of the mammary gland to the stress of lactation appears to occur as two distinct phases. The first, occurring soon after parturition, involves the sudden rise in the number of functional cells as a result of the wave of mitosis and may be regarded as the immediate response. The second is the slower change which occurs during lactation itself and is more probably related to the increasing demands on the gland by the growing pups.

SUMMARY

1. Changes in the total nitrogen, mitochondrial nitrogen, total ribonucleic acid (RNA), mitochondrial RNA and in the deoxyribonucleic acid (DNA) content of the rat mammary gland have been studied at intervals during the lactation cycle.

2. There is a rise in the total nitrogen of the gland through pregnancy and lactation; a similar rise is found in the mitochondrial nitrogen.

3. RNA increases progressively from early pregnancy to late lactation and declines again during mammary involution. Parturition is accompanied by a rapid rise in the RNA of the mammary gland. The mitochondrial RNA rises during pregnancy, but reaches a plateau value by the third day of lactation which is maintained until mammary involution sets in.

4. The DNA of the gland increases during pregnancy. There is a doubling of the DNA level at parturition after which it remains constant throughout lactation.

5. Changes in the level of RNA are discussed in relation to the rate of protein synthesis in the gland and evidence is adduced for an increase in the microsomal and submicrosomal fractions during lactation.

6. The significance of the changes in DNA content of the mammary gland are discussed. It is suggested that the changes in DNA content of the gland at parturition indicate that a wave of cell division occurs in the gland around that time.

We wish to acknowledge an expenses grant from the A.R.C. to one of us (A. L. G.).

REFERENCES

Albaum, H. G. & Umbreit, W. W. (1947). *J. biol. Chem.* **167**, 369.

Brachet, J. (1955). In *The Nucleic Acids*. Ed. by Chargaff, E. and Davidson, J. N. New York: Academic Press Inc.

Davidson, J. N. (1953). *The Biochemistry of the Nucleic Acids*, 2nd ed. London: Methuen and Co.

Davidson, J. N. & Leslie, I. (1950). *Nature, Lond.*, **165**, 49.

Dische, Z. (1955). In *The Nucleic Acids*. Ed. by Chargaff, E. and Davidson, J. N. New York: Academic Press Inc.

Duve, C. de & Berthet, J. (1953). *Nature, Lond.*, **172**, 1142.

Folley, S. J. & Greenbaum, A. L. (1947). *Biochem. J.* **41**, 261.

Fukuda, M. & Sibatani, A. (1953). *J. Biochem., Tokyo*, **40**, 95.

Greenbaum, A. L. & Slater, T. F. (1957*a*). *Biochem. J.* **66**, 148.

Greenbaum, A. L. & Slater, T. F. (1957*b*). *Biochem. J.* **66**, 161.

Hevesy, G. & Ottesen, J. (1943). *Acta Physiol. scand.* **5**, 237.

Hogeboom, G. H., Schneider, W. C. & Palade, G. E. (1948). *J. biol. Chem.* **172**, 619.

Howe, A., Richardson, K. C. & Birbeck, M. S. C. (1956). *Expt. Cell Res.* **10**, 194.

Jeffers, K. R. (1935). *Amer. J. Anat.* **50**, 257.

Kirkham, W. R. & Turner, C. W. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 123.

Leslie, I. (1955). In *The Nucleic Acids*. Ed. by Chargaff, E. and Davidson, J. N. New York: Academic Press Inc.

Maeder, L. M. A. (1922). *Amer. J. Anat.* **31**, 1.

Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.

Roberts, F. L. (1921). *Pap. Mayo Fdn med. Educ.* I. Philadelphia: W. B. Saunders and Co.

Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.

Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.

Schneider, W. C. (1946). *J. biol. Chem.* **164**, 747.

Schneider, W. C. & Hogeboom, G. H. (1951). *Cancer Res.* **11**, 1.

Smellie, R. M. S., McIndoe, W. M., Logan, R., Davidson, J. N. & Dawson, I. M. (1953). *Biochem. J.* **54**, 280.

Thomson, R. Y., Heagy, F. C., Hutchison, W. C. & Davidson, J. N. (1953). *Biochem. J.* **53**, 460.

Vendrely, R. (1955). In *The Nucleic Acids*. Ed. by Chargaff, E. and Davidson, J. N. New York: Academic Press Inc.

Weatherford, H. L. (1929). *Amer. J. Anat.* **44**, 199.

Studies on the Particulate Components of Rat Mammary Gland

3. THE RELATIONSHIP BETWEEN ENZYME ACTIVITY AND PARTICLE COUNTS IN MAMMARY GLAND SUSPENSIONS

By A. L. GREENBAUM and T. F. SLATER
Department of Biochemistry, University College, London

(*Received* 15 *November* 1956)

The two preceding parts of this series (Greenbaum & Slater, 1957*a*, *b*) have reported the rapid changes in chemical content which occur in the rat mammary gland over the period of parturition. This paper describes changes in the enzymic activity of the gland throughout the lactation cycle.

Part 1 of this series showed that the increase in tissue nitrogen at parturition was both rapid and considerable, and Part 2 described the rapid increases in milk synthesis and nucleic acid content which occur over this same period. Thus the gland in late pregnancy contrasts vividly, both in secretory activity and in composition, with the gland a few days later when lactation is well established.

It is to be expected that the transition of the gland into the 'active' state would be accompanied by an increase in the activity of the oxidative-enzyme systems. That such an increase occurs is evidenced by the rapid rise in Q_{O_2} and R.Q. (Folley & French, 1949), the increases in succinic oxidase and cytochrome oxidase activity (Tuba, Orr & Wiberg, 1955) and in glucose 6-phosphate and 6-phosphogluconate dehydrogenases (Glock & McLean, 1954).

Recent investigations have localized the majority of the oxidative enzymes in the particles of mitochondrial size (see Schneider, 1956). It seemed of interest, therefore, to follow in mammary gland suspensions the alterations in the activity of an enzyme which, in other tissues, is known to be associated with the mitochondrial fraction. In particular, it was of interest to see whether the rapid increase in synthetic activity found in the gland *post partum* was associated with changes in the number of mitochondrial particles, an increase in size of the existing particles or an increasing efficiency of the enzyme systems.

It has repeatedly been shown, in diverse tissues, that succinic oxidase is almost completely localized in the mitochondrial fraction. Although this enzyme has been studied in mammary tissue (guinea pig,

Moore & Nelson, 1952; rat, Tuba *et al.* 1955; mouse, Dmochowski & Strickland, 1953) it was thought that a re-investigation into the variations in activity in the full homogenate and in the mitochondrial fraction over the lactation cycle would be of interest and would provide information on the following points: (i) the intracellular localization of the enzyme at various stages of the lactation cycle; (ii) the variations in the total activity of the enzyme in the whole-tissue suspension and in the mitochondria at various stages of the lactation cycle; (iii) variation in the recovery of the enzyme in the mitochondrial fraction, under standard conditions of centrifuging, during the lactation cycle; (iv) the correlation between these variations and the changes in ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and nitrogen which occur during the lactation cycle.

In order to carry the correlation of enzymic activity with mitochondrial fractions a stage further, counts were made of particle numbers in the original tissue suspension and in the mitochondrial suspension derived from it. These counts have been used to correlate recoveries of mitochondrial nitrogen, RNA and enzymic activity with the number of particles recovered.

METHODS

Animals. The animals used and the method of distribution of the rats into the various groups has already been described, as has the method of preparing the tissue suspension and the chemical procedures for lactose content, deoxyribonucleic acid phosphorus (DNA-P), ribonucleic acid phosphorus (RNA-P) and total nitrogen content of the gland (Greenbaum & Slater, 1957*b*).

Measurement of succinic oxidase activity. A method for the determination of succinic oxidase activity has been described by Schneider & Potter (1943). In most tissues, such as liver and kidney, this assay presents no difficulty, but in mammary gland the activity of the enzyme system is inhibited by the presence of a high endogenous level of trace metals in the milk and tissue. The Cu^{2+} ion concentration in rat milk may reach 7 μg./ml. ($1{\cdot}1 \times 10^{-4}$ M; Cox & Mueller, 1937), and considerable quantities of iron, calcium and zinc are also present. Succinic oxidase is strongly inhibited by trace concentrations of heavy metals (Hirade & Hayashi, 1953), although such inhibition can be removed by the addition of ethylenediaminetetra-acetic acid (EDTA) (Altmann & Crook, 1953). The addition of chelating agents is therefore of prime importance in enzyme assays on mammary material. Experiments on the activity of succinic oxidase in mammary tissue suspensions from both pregnant and lactating rats in the presence of variable concentrations of EDTA showed that maximum enzymic activity occurred with a final concentration of 0·003 M. The conditions for the assay procedure used in this communication were as follows: 1 ml. of 0·1 M-phosphate buffer, pH 7·4; 0·3 ml. of 0·5 M-succinate, pH 7·4; 0·2 ml. of 2×10^{-4} M-cytochrome *c*; 0·1 ml. of 0·1 M-EDTA, pH 7·4; 0·4, 0·7 or 1 ml. of 1:10 tissue or mitochondrial suspension; 0·2 ml. of 20% KOH in centre well and water to 3 ml. Temperature, 37°. Readings were taken at 10 min. intervals for 30 min. The succinic oxidase activity of mitochondrial and tissue suspensions remained constant for several hours when stored on ice.

Mitochondrial counting procedure. Counting, as a means of quantitative assay, has only recently been developed for use with mitochondrial suspensions (Allard, Mathieu, Lamirande & Cantero, 1952; Shelton, Schneider & Striebich, 1953; Agrell, 1955). In the present experiments particulate counts on suspensions of mammary tissue and on large-particle suspensions obtained by the differential centrifuging of these suspensions have been carried out by the following modification of the procedure described by Allard *et al.* (1952), with dark-ground illumination. The tissue or mitochondrial suspension was diluted by the addition of a calculated volume of ice-cold saturated (1·8 M) sucrose. The volume added was so adjusted that each small square of the counting chamber contained about ten particles. The diluted suspension was then shaken vigorously and mounted in a Helber-type bacterial counting chamber with a grid-ruling of Thoma construction. The count was made with a dark-ground zonal condenser at a final magnification of $\times 1000$. All visible particles up to and including the mitochondrial size range were counted. In general, sixteen of the sixty-four large squares of the grid were counted, the sixteen squares being symmetrically distributed. To avoid bacterial contamination the 1·8 M-sucrose was always prepared immediately before use and, as a check, counts on the suspending medium were always performed and used as blanks to correct the particle counts.

The hypertonicity of the saturated sucrose solution caused all particles to assume a spherical shape and this made it exceedingly difficult to differentiate the particles of different size ranges. Further, the use of dark-ground illumination permitted the detection and counting of much smaller particles than would have been possible with transmitted light. As a result of these two factors the counting procedure included particles much smaller than those usually counted as mitochondria. Thus the 'large-particle fraction' counted here includes both large and small mitochondria, and perhaps also a proportion of the larger type of microsomal particle, but excludes small microsomes. The wide range of particles counted is not, perhaps, wholly disadvantageous since it includes small mitochondria and large microsomes, fractions which have been shown to change under conditions of physiological or abnormal stress (Gavosto & Moyson, 1953; Thomson & Mikuta, 1954; Thomson & Moss, 1955; Greenbaum, Slater & Reid, 1955). The counting procedure appears to be accurate to $\pm 10\%$. In one typical count the sum of the counts of the particles in the separate nuclear, mitochondrial and supernatant fractions of a unit volume of suspension was 1009, while the count of the same volume of original suspension, from which these were prepared, was 905.

RESULTS

Mitochondrial counts

The results of particle counts obtained in mammary tissue and mitochondrial suspensions are given in Table 1, together with the percentage of the total suspension count sedimented in the large-particle fraction.

Table 1. *Large-particle counts in suspensions of rat mammary tissue*

Status	No. of animals	10^{-12} × Particle count/100 g. wet tissue: Whole suspension	10^{-12} × Particle count/100 g. wet tissue: Mitochondrial suspension	Recovery of large particles in the mitochondrial fraction (%)	10^{-12} × Homogenate count*: DNA-P	10^{-12} × Homogenate count*: Mitochondrial N	10^{-12} × Homogenate count*: Tissue N
Virgin	3†	103	—	—	21·4	12·6	0·39
Pregnant (20 days)	8	1240 ± 150‡	890 ± 110	34 ± 9	97·3 ± 19·2	18·9	6·78
Lactating (3 days)	7	151 ± 38	73 ± 14	62 ± 8	7·6 ± 2·2	1·08	0·23
Lactating (18 days)	6	100 ± 22	64 ± 12	69 ± 5	4·9 ± 1·5	0·37	0·14
Involuting (2 days)	3	172 ± 10	105 ± 12	61 ± 6	14·1 ± 5·4	0·94	0·15

* Units: $\frac{\text{Count/100 g. wet wt. of gland}}{\text{mg./100 g. wet wt. of gland}}$.

† Pooled sample obtained from three animals.

‡ In all tables the values given are group means ± standard error of the mean.

It can be seen that the number of particles/g. of milk-free mammary tissue rises to a maximum at the end of pregnancy. The number falls rapidly over the period of parturition and then remains approximately constant over the remainder of lactation. The mean percentage recovery of the particles, about 60 %, is approximately constant at all stages of the lactation cycle examined. If it is assumed that the number of cells in the tissue is directly proportional to the DNA content (see Greenbaum & Slater, 1957*a*) then the number of particles counted per cell is indicated by the ratio count:DNA-P content of the suspension (Table 1, column 6).

The table shows that the number of particles per cell rises to a maximum at the end of pregnancy and then falls precipitously over parturition.

Variations in succinic oxidase activity

The results of the estimations of succinic oxidase activity in mammary tissue suspensions are shown in Table 2. The table also shows the percentage of the activity sedimented in the large-particle fraction.

There is a similar pattern of change of succinic oxidase activity in both tissue and mitochondrial suspensions. In both there is a sharp rise in activity at parturition and maximum activity is reached in the second half of lactation. Table 2 also includes the activity of succinic oxidase calculated in terms of the total-nitrogen content of the mitochondrial fraction. If a fixed amount of enzyme activity were associated with a unit quantity of mitochondrial nitrogen then the ratio of activity to nitrogen would be constant throughout the lactation cycle. The ratio, however, varies considerably, which indicates that succinic oxidase activity and mitochondrial nitrogen can vary independently. Although cell numbers are practically doubled over parturition (Greenbaum & Slater, 1957*a*, *b*) the ratio of succinic oxidase activity:DNA-P rises at parturition (Table 2, column 9) which suggests a build up in the oxidative system per cell.

The percentage of the total activity sedimented in the large-particle fraction rises from 55 % in late pregnancy to 85 % at the end of lactation. The activity per particle counted in the large-particle fraction varies over the lactation cycle as shown by the ratio of succinic oxidase activity:particle count (Table 2, column 11).

DISCUSSION

Parturition is so obviously a transition period leading to a higher level of metabolic and synthetic activity in the mammary gland that the rapid decrease in particles counted per gram of tissue over this period is most striking and unexpected.

Table 2. *Changes in succinic oxidase activity in rat mammary gland*

Status	Activity in whole homogenate: No. of animals	$10^{-3} \times \mu$l. of O_2 uptake/hr./100 g. wet wt. of gland	μl. of O_2 uptake/hr./mg. of N	Activity in mitochondrial suspension: No. of animals	$10^{-3} \times \mu$l. of O_2/hr./100 g. wet wt. of gland	μl. of O_2/hr./mg. of N
Pregnant (20 days)	9	34±4	65·5±5·0	8	17±3·9	216±51
Lactating (3 days)	10	101±5·8	98·0±9·0	9	74±6·3	580±97
Lactating (10 days)	6	144±19	160±19	6	113±16·4	534±75
Lactating (18 days)	8	189±20	132±17	6	144±10·5	411±52
Involuting (2 days)	3	46±10·7	33±4	2	37±7·5	167
Involuting (4 days)	2	28	44	—	—	—

Status	Percentage of total activity in mitochondrial fraction	Homogenate activity* DNA-P†	Homogenate activity* Tissue N†	Activity of homogenate* Count†
Pregnant (20 days)	55±12	1499±660	184	22·7±8·8
Lactating (3 days)	71±5	4950±586	150	1044±260
Lactating (10 days)	76±6	7200±970	236	—
Lactating (18 days)	85±8	8930±1260	266	2618±605
Involuting (2 days)	84	3251±1030	39·7	266±59
Involuting (4 days)	—	180±39	—	—

* Units: μl. of O_2 uptake/hr./100 g. wet wt. of gland.

† Units: DNA-P in mg./100 g. wet wt. of gland. Tissue N in mg./100 g. wet wt. of gland. Count as $10^{-12} \times$ no. of particles/100 g. wet wt. of gland.

‡ Pooled sample obtained from three animals.

The fall in particle numbers at parturition is associated in time with increases in tissue nitrogen, RNA-P, DNA-P and of enzymic activity. Metabolic processes are so intimately associated with the particulate material that it seems unlikely that the particle content of the cell should be built up at a time when metabolism is relatively low and then dissipated into the very small submitochondrial range when the need is greater. It is not possible at this stage to decide whether the particles dissociate, whether they coalesce to form larger particles or whether the decrease results from a decrease in the large vesicular system of the cell. But whatever the explanation it seems fairly certain from the data presented here and in the previous paper (Greenbaum & Slater, 1957*b*) that at parturition there is a redistribution of the material between the intracellular fractions of the gland.

Succinic oxidase has been shown to be linked with mitochondria in a great variety of tissues other than rat mammary gland. The percentage sedimentation of the succinic oxidase activity is closely correlated with the percentage of particles recovered in the large-particle fraction. The mean percentage of succinic oxidase activity sedimented in late lactation is 85% and from this, and from the fact that changes in enzyme activity of the whole tissue suspension are exactly duplicated in the mitochondrial enzyme activity, it appears that succinic oxidase is wholly linked to the large-particle fraction sedimenting at 230000 ***g*** min. in isotonic sucrose. However, the fact that 230000 ***g*** min. is required to sediment the greater part of the total succinic oxidase activity (smaller centrifugal fields give smaller recoveries) indicates that the average size of the particles containing succinic oxidase is smaller in the mammary gland than in the liver, where 50000 ***g*** min. is sufficient to sediment 60% of the total activity (Schneider & Hogeboom, 1950). A similar conclusion may be inferred from the results previously reported by Dmochowski & Strickland (1953) on mouse mammary tissue.

The low level of succinic oxidase activity in the virgin tissue is an indication of the small metabolic requirement of a tissue which is synthetically non-functional. The onset of secretion, with the consequent demand for a tissue with highly developed and active enzyme systems witnesses a sharp rise in succinic oxidase activity which is carried on throughout lactation. In involution the activity falls precipitously. The pattern of change of succinic oxidase activity described here is different from that presented by Moore & Nelson (1952) who, however, worked on guinea pigs. In the guinea pig lactation is fully established almost immediately after parturition and then decreases throughout the remainder of lactation. In the rat lactation reaches its maximum near the end of the period of lactation (Brody & Nisbet, 1938). Thus the increasing succinic oxidase activity in lactation is in agreement with a view linking the activity of oxidative enzyme systems with milk synthesis.

The increase in succinic oxidase activity from late pregnancy to mid-lactation could be due to any of the following effects: (i) an increase in mitochondrial numbers with no increase in size or enzyme activity per particle; (ii) an increase in mitochondrial size, which, assuming the preservation of typical mitochondrial enzyme patterns, would lead to an increase in the amount of activity associated with each mitochondrion; (iii) an increase in the activity of the succinic oxidase system itself; (iv) any combination of the foregoing three possibilities.

There is little doubt that there is an increase in the number of mitochondria in the gland from late pregnancy to late lactation. Weatherford (1929) observed, cytologically, an increased number of mitochondria per cell over lactation and, when this is coupled with the doubling of cell numbers shortly after parturition (Greenbaum & Slater, 1957*a*), it is obvious that the total number of mitochondria in the gland must increase substantially over pregnancy and lactation. This is further indicated by the rapid rise in mitochondrial nitrogen (Greenbaum & Slater, 1957*b*), and quantitative counts by Howe, Richardson & Birbeck (1956) on guinea-pig tissue also lead to the view that mitochondrial numbers increase after parturition. This would imply that simultaneously with the increase in mitochondrial numbers there must be a decline in the number of particles other than mitochondria counted under the dark-ground conditions to account for the over-all fall in numbers observed.

Observations of an increased size of mitochondria in early lactation compared to late pregnancy have also been reported (Weatherford, 1929; Jeffers, 1935), with a change in shape from spheres predominantly in pregnancy to filaments and rods in lactation. The increasing sedimentation of the succinic oxidase in the large-particle fraction can thus be considered to indicate an increase in size of the particles containing succinic oxidase. Changes in size of mitochondria after stress have also been reported by Thomson & Mikuta (1954) and Thomson & Moss (1955).

No activators for the succinic oxidase system have, as yet, been reported (see Bonner, 1955), whereas many substances act as potent inhibitors. Metal ions, oestrogens, and mercaptide-forming agents have been reported to inhibit the succinic oxidase system. Of these, metal ions may be potent inhibitors in the mammary gland as they probably accumulate in the mammary gland during pregnancy; colostrum has, in general, a much higher content of these metal ions than milk secreted in active lactation (see Underwood, 1956). The increase in succinic oxidase activity which occurs over parturition may be due in part to the removal of some of these metal ions from the gland by the secretion of milk. The copper content of rat milk is high when compared with other tissues [2–3 μg./g. of rat liver (Cunningham, 1931) and 7 μg./ml. of milk (Cox & Mueller, 1937)] and, if the rat shows a similar pattern of change to all other species examined, colostrum should have an even greater content of copper than 7 μg./ml. Thus the initiation of lactation may result in the removal of metal-ion inhibitors and hence in an increased enzyme activity.

The influence of inhibitory agents may be evaluated approximately from the following considerations. The ratio of succinic oxidase activity to mitochondrial nitrogen rises at parturition (Table 2, column 7). This cannot be interpreted in terms of an increase either of number or size if it is assumed that the nitrogen content per unit of mitochondrial volume does not alter significantly at this period. The rise in the ratio must be interpreted in terms of the activity increasing more rapidly than the mass, which suggests that the effect is due to the removal of inhibitors. This effect accounts for some 50 % of the total rise in succinic oxidase activity at parturition.

Reasons can, therefore, be advanced for the operation of each of the three mechanisms proposed to account for the rise in succinic oxidase activity.

The results given here and in the previous paper (Greenbaum & Slater, 1957*b*) give a more precise picture of the mechanism by which the mammary gland responds to the increasing demands imposed on it by lactation. After the initial preparatory burst of mitosis there is a more protracted change in the nature of the cellular inclusions. It seems that advancing lactation causes a doubling in the amount of mitochondrial substance and probably a change in size towards larger particles. It is likely that a study of the changes in enzyme content of the different components of the mitochondrial population as lactation advances would throw more light on this adaptation.

SUMMARY

1. Succinic oxidase activity in suspensions of rat mammary tissue increases sharply at parturition and reaches a maximum value midway through lactation. The identical patterns of change of activity in the whole-tissue suspension and in the large-particle fraction, together with the high recoveries of activity in the large-particle fraction, indicate that the succinic oxidase activity of rat mammary tissue is very largely localized in this fraction.

2. The increase in succinic oxidase activity at parturition and in early lactation is probably the result of three effects: (i) an increase in mitochondrial numbers; (ii) an increase in mitochondrial size; (iii) the removal of inhibitory factors at parturition.

3. The large-particle count procedure indicated a rapid decrease in the number of particles present over parturition. It is suggested that this was the result of a rapid decrease in the number of particles other than mitochondria occurring simultaneously with a smaller increase in mitochondrial numbers.

4. Interrelationships between ribonucleic acid, deoxyribonucleic acid, tissue nitrogen, succinic oxidase and the large-particle counts are discussed.

We are greatly indebted to the Agricultural Research Council for an expenses grant to one of us (A.L.G.).

REFERENCES

Agrell, I. (1955). *Exp. Cell Res.* **8**, 232.
Allard, C., Mathieu, R., Lamirande, G. de & Cantero, A. (1952). *Cancer Res.* **12**, 407.
Altmann, S. M. & Crook, E. M. (1953). *Nature, Lond.*, **171**, 76.
Bonner, W. D. (1955). In *Methods in Enzymology*, vol. 1. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Brody, S. & Nisbet, R. N. (1938). *Res. Bull. Mo. agric. Exp. Sta.* no. 285.
Cox, W. M. jun. & Mueller, A. J. (1937). *J. Nutr.* **13**, 249.
Cunningham, I. J. (1931). *Biochem. J.* **25**, 1267.
Dmochowski, L. & Strickland, L. H. (1953). *Brit. J. Cancer*, **7**, 250.
Folley, S. J. & French, T. H. (1949). *Biochem. J.* **45**, 117.
Gavosto, F. & Moyson, F. (1953). *Experientia*, **9**, 263.
Glock, G. E. & McLean, P. (1954). *Biochem. J.* **56**, 171.
Greenbaum, A. L. & Slater, T. F. (1957*a*). *Biochem. J.* **66**, 148.
Greenbaum, A. L. & Slater, T. F. (1957*b*). *Biochem. J.* **66**, 155.
Greenbaum, A. L., Slater, T. F. & Reid, E. (1955). *Nature, Lond.*, **175**, 506.
Hirade, J. & Hayashi, T. (1953). *J. Biochem., Tokyo*, **40**, 461.
Howe, A., Richardson, K. C. & Birbeck, M. S. C. (1956). *Exp. Cell Res.* **10**, 194.
Jeffers, K. R. (1935). *Amer. J. Anat.* **56**, 257.
Moore, R. O. & Nelson, W. L. (1952). *Arch. Biochem. Biophys.* **36**, 178.
Schneider, W. C. (1956). In *Proc. 3rd Int. Congr. Biochem., Brussels.* New York: Academic Press Inc.
Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
Schneider, W. C. & Potter, V. R. (1943). *J. biol. Chem.* **149**, 217.
Shelton, E., Schneider, W. C. & Striebich, M. J. (1953). *Exp. Cell Res.* **4**, 32.
Thomson, J. F. & Mikuta, T. (1954). *Arch. Biochem. Biophys.* **51**, 487.
Thomson, J. F. & Moss, E. M. (1955). *Cancer, N.Y.*, **8**, 789.
Tuba, J., Orr, P. F. & Wiberg, G. S. (1955). *Canad. J. Biochem. Physiol.* **33**, 904.
Underwood, E. J. (1956). In *Trace Elements in Human and Animal Nutrition.* New York: Academic Press Inc.
Weatherford, H. L. (1929). *Amer. J. Anat.* **44**, 199.

Studies on Pituitary Adrenocorticotropin

2. OXIDATION–REDUCTION PROPERTIES OF THE HORMONE*

By M. L. DEDMAN, T. H. FARMER and C. J. O. R. MORRIS
Department of Experimental Biochemistry, London Hospital Medical School, London, E. 1

(*Received* 27 *August* 1956)

It is generally accepted that the biological activity of the pituitary adrenocorticotropic hormone (corticotropin, ACTH) is unstable under oxidizing conditions. For example, Dixon, Moore, Stack-Dunne & Young (1951) have advocated the use of anti-oxidants to diminish oxidative inactivation during extraction and purification of the hormone, and the practice has often been adopted by later workers. No quantitative data on the subject are, however, available.

The first evidence that the oxidation process may be reversible, at least in part, was provided by Kuehl, Meisinger, Brink & Folkers (1953). These workers showed that corticotropin B, a highly active preparation made by partial peptic hydrolysis of purified pituitary extracts, could be oxidized by the action of oxygen in the presence of ferric iron, to a product which was inactive in the ascorbic acid-depletion bioassay. The biological activity of the product could be restored by several days' contact with hydrogen sulphide. Attempts in several laboratories to repeat the reductive re-activation of oxidized hormone under the conditions advocated by Kuehl *et al.* (1953) have, however, been unsuccessful (Dixon & Stack-Dunne, 1955).

The observations of Kuehl *et al.* (1953) are of interest from both the chemical and biological standpoints. First, it is generally agreed that the best corticotropin preparations now available contain no detectable sulphydryl groups, so that there is no obvious chemical explanation of the oxidation–reduction property. Secondly, the presence of an oxidation–reduction centre may also be of importance for the biological function of a

* Part 1: Cortis-Jones, Crooke, Henly, Morris & Morris (1950).

hormone which controls an oxidative process, the oxidation of cholesterol in the adrenal cortex to C_{19} and C_{21} precursors of the adrenal hormones.

The present work has been concerned with the determination of the optimum conditions for the oxidation and reduction of corticotropin. Some observations on the nature of the oxidation–reduction centre in the hormone are reported. A preliminary account of part of this work has already been published (Dedman, Farmer & Morris, 1955).

EXPERIMENTAL

Bioassay methods

Adrenocorticotropic potency has been measured by the adrenal ascorbic acid-depletion method of Sayers, Sayers & Woodbury (1948) with minor modifications (Morris, 1951).

The particular corticotropin preparation used for chemical treatment was always bioassayed in the untreated state, together with the treated material, so that any change in biological potency brought about by the chemical treatment can be expressed as a percentage of the initial potency. The absolute potencies of many of the untreated preparations assayed against international standard corticotropin are also given.

The values given in Tables 2–4 are the best estimates of potency, the confidence limits of the estimate at P 0·95, calculated according to Pugsley (1946), being given in parentheses.

Reagents

Analytical grade acetone was dried over anhydrous K_2CO_3 for 24 hr. and redistilled, the fraction of b.p. 56–57° being collected. Analytical grade acetic acid (2 l.) was mixed with water (50 ml.) and was boiled under reflux for 3 hr. The product was distilled through a 30 cm. × 2 cm. fractionating column packed with glass Fenske spirals and the fraction of b.p. 117–118° was collected. Ether (analytical grade) was freed from peroxides immediately before use by passage through a 20 cm. × 2·5 cm. column packed with Al_2O_3 (grade H, P. Spence and Co., Widnes).

Oxycellulose. This, with 10% carboxyl content, was obtained from the Tennessee Eastman Co., Kingsport, Tenn., U.S.A. It was purified immediately before use by the method of Astwood, Raben, Payne & Grady (1951) by washing with N-HCl for 10 min., followed by water and finally with 0·1 N-acetic acid for 10 min.

Resins. Cation-exchange resin Amberlite XE-97, 200–400 mesh (Chas. Lennig and Co., 18 York Buildings, London, W.C. 2), is a finely divided preparation of the carboxylic acid resin Amberlite IRC-50, and is also chemically identical with Amberlite XE-64. It was treated according to the method of Hirs, Moore & Stein (1953) to remove soluble and very fine material and was subsequently dried to constant weight over silica gel *in vacuo*. The anion-exchange resins Amberlite IR-4B and Amberlite IRA-400, 60–100 mesh (Chas. Lennig and Co.), were treated in sequence with 2N-HCl, water and 2N-Na_2CO_3 for three successive cycles, and were stirred for 30 min. with each reagent. The resins were finally converted into the acetate forms by treatment with 2N-acetic acid. Dowex 50 × 8, 200–400 mesh, and Dowex 50 × 16, 60–100 mesh, cation-exchange resins (Dow Chemical Co., Midland, Mich., U.S.A.) were treated with 4N-HCl and hot N-NaOH to remove soluble material according to the method of Moore & Stein (1951). Dowex 2 × 8, 60–100 mesh, anion-exchange resin (Dow Chemical Co.) was treated similarly to Amberlite IR-4B.

Preparation of crude corticotropin

The corticotropin preparations used were prepared from the anterior lobes of pig pituitary glands by minor modifications of published methods. Because of the wide variations in yields and potencies reported in the literature, a detailed description of the preparation is appended.

Whole pig pituitary glands, stored in anhydrous acetone, were received in the laboratory within five days of the death of the animal. The glands were stored for a further 24 hr. in fresh anhydrous acetone and the anterior lobes separated. These were thoroughly homogenized with anhydrous acetone in a high-speed blender and the homogenate was centrifuged. The precipitate was dried *in vacuo*. The dry powder assayed at 0·2–0·3 i.u./mg. Experience has shown that it is not possible to obtain high-potency preparations from a pituitary-gland powder assaying less than 0·2 i.u./mg.

The pituitary powder was extracted with acetic acid by the method of Payne, Raben & Astwood (1950) or the method of Dedman, Farmer, Morris & Morris (1952). Both methods give products with potencies of 1–1·8 i.u./mg. It is particularly important that the acetic acid used should be freed from trace amounts of acetic anhydride as described under Reagents. The ether precipitate should be very thoroughly washed free from acetic acid with anhydrous acetone and dried *in vacuo* over silica gel.

Purification with oxycellulose. This step was carried out according to the procedure of Astwood *et al.* (1951). The optimum amount of oxycellulose required per unit weight of crude corticotropin varies with different preparations of the latter and can only be found from trial experiments. The ratio appears to be quite reproducible for the same preparations of corticotropin and oxycellulose. The biological activities of oxycellulose concentrates prepared in this laboratory have been in the range 30–70 i.u./mg. but have seldom been less than 40 i.u./mg.

Ion-exchange chromatography. The method used was a modification of that of White & Fierce (1953). The purified Amberlite XE-97 resin (20 g.) was mixed with N-NaOH (184 ml.) and M-NaCl (20·4 ml.), stirred for 1 hr. and allowed to stand for 48 hr. at room temperature. The pH of the supernatant was then 8·3. A column 30 cm. × 2 cm. was prepared by pouring the resin slurry into a suitable glass chromatographic tube in six equal portions. The column was then washed with 2–3 l. of 0·1 M-$NaHCO_3$. The pH of the effluent liquid was then identical with that of the 0·1 M-$NaHCO_3$ wash liquid, 8·32. The upper 1–2 cm. of the resin was gently stirred and allowed to settle slowly in order to produce a flat upper surface.

An oxycellulose concentrate of corticotropin (100 mg.) was dissolved in water (10 ml.) and 0·2 M-$NaHCO_3$ (10 ml.) added dropwise with stirring. After a further 20 min. stirring the mixture was centrifuged and the supernatant liquid transferred to the column. The flow rate was adjusted so that the solution entered the column during the course of about 2 hr. Chromatographic development was continued with 0·1 M-$NaHCO_3$ at a flow rate of 15 ml./hr. Fractions (3 ml.) were collected by means of an automatic fraction collector, each fraction being taken into a tube containing 0·05 ml. of acetic acid, in order to reduce the period during

which the corticotropin was exposed to alkaline conditions. The ultraviolet absorption of each fraction was measured at 275 mμ in 1 cm. quartz cells with a Unicam SP. 500 spectrophotometer. Fig. 1 shows a typical separation obtained in this way.

Three well-defined components have been found. These appear to correspond with the corticotropin fractions A_1, A_2 and A_4 of Dixon & Stack-Dunne (1955), obtained by chromatography on a similar ion-exchange resin at pH 6·7 and at a higher ionic strength. Accordingly their nomenclature has been adopted for these components.

Corticotropins A_1 and A_2 were obtained from the $NaHCO_3$ solutions by a combination of ion-exchange desalting and oxycellulose adsorption.

In a typical experiment, 135 ml. of 0·1 M-$NaHCO_3$ containing corticotropin A_1 was adjusted to pH 3·5 with concentrated HCl and passed through a 9·8 cm. × 1·3 cm. column containing 7 g. of Dowex 50 × 16 cation-exchange resin in the H form. The column was washed with 20 ml. of 0·1 N-acetic acid and Cl^- ions were removed from the total effluent by passage through an 8·3 cm. × 1·3 cm. column containing 4 g. of Amberlite IR-4 B anion-exchange resin in the acetate form. The corticotropin in the chloride-free solution was adsorbed on to oxycellulose (150 mg.). Adsorption was complete after stirring for 20 hr. at room temperature. The oxycellulose was separated by centrifuging, washed with 0·1 N-acetic acid and the corticotropin eluted by shaking with 0·1 N-HCl (3 ml.) for 1 hr. The oxycellulose was eluted with three further 1 ml. portions of 0·1 N-HCl for 2 min. periods. The combined eluates were freed from HCl by passage through a 7·2 cm. × 0·7 cm. column of Dowex 2 × 8 anion-exchange resin (1·5 g.) in the acetate form and freeze-dried. The yield of corticotropin A_1, based on the ultraviolet absorption at 275 mμ of the original solution, was 83%. The recovery of biological activity has always been in close agreement with the weight recovery. The process of re-adsorption on oxycellulose described above appears to improve considerably the stability of the product.

Biological activity. Preparations of corticotropin A_1 made by this procedure have a biological activity in the range 75–150 i.u./mg., depending mainly on the potency of the oxycellulose concentrate subjected to chromatography. The majority of the preparations used in this work had a potency greater than 90 i.u./mg. Corticotropin A_2 preparations have shown lower biological activities in the range 38–68 i.u./mg. Dixon & Stack-Dunne (1955) also found lower potencies for the corresponding fractions. Rechromatography of corticotropins A_1 and A_2 under conditions similar to those employed for their separation has shown consistent behaviour and has confirmed that the resolution of the two components is essentially complete. The potencies and yields of the various fractions obtained in the course of a typical corticotropin preparation are given in Table 1.

The work described in this paper has been carried out either with oxycellulose concentrates (OC) or with corticotropin A_1. The existence of a reversible oxidation–reduction system has, however, been confirmed in corticotropin A_2, and in corticotropin preparations which had been partially hydrolysed with HCl. The work of Kuehl *et al.* (1953) demonstrates the existence of a similar system in pepsin-hydrolysed corticotropin.

Table 1. *Purification of corticotropin*

Stage	Potency (i.u./mg.)	Yield (g.)
Acetone-dried anterior pituitary powder	0·20–0·30	100
Crude corticotropin	1·0–1·8	10–11
Oxycellulose concentrate	40–50	0·2–0·3
Corticotropin A_1	90–110	0·06–0·1
Corticotropin A_2	40–60	0·02–0·04

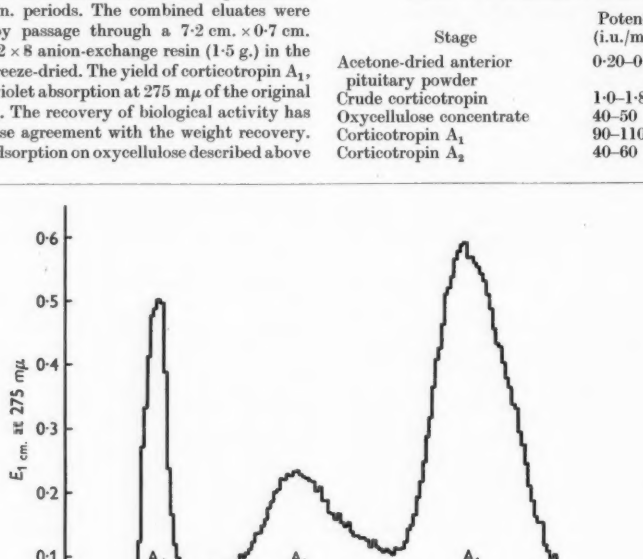


Fig. 1. Chromatography of an oxycellulose concentrate of corticotropin on Amberlite XE-97 cation-exchange resin, 0·1 M-$NaHCO_3$, 3 ml. fractions.

Amino acid determinations

Hydrolysis of corticotropin preparations was carried out with redistilled 20% HCl (constant b.p.) in sealed tubes for 18 hr. at 115°.

Quantitative estimation of the amino acids was carried out either by paper chromatography or by ion exchange. In the first method the amino acids were separated in a one-dimensional chromatogram with the phenol–buffer, pH 12, or *m*-cresol–buffer, pH 8·4, system of McFarren (1951). Standards (5, 10 and 15 μg.) of the particular amino acids to be estimated were chromatographed simultaneously on the same strip. After removal of the solvent the amino acid spots were developed with ninhydrin according to the method of Wael & Cadavieco (1954). The coloured zones were cut out and the pigment was eluted with 50% (v/v) aqueous propan-1-ol. After removal of the filter paper, the optical densities of the solutions were determined at 570 mμ in a Unicam SP. 600 spectrophotometer. The amount of amino acid in the portion of hydrolysate was determined by comparison with standards which were chromatographed and developed simultaneously.

Complete amino acid analyses of a typical preparation of corticotropin A_1, and of peroxide-oxidized material prepared from it, were carried out with the method of ion-exchange chromatography on Dowex 50 × 8 resin (Moore & Stein, 1951), together with the improved photometric ninhydrin method of Moore & Stein (1954). Unsatisfactory recoveries of arginine were obtained with the 15 cm. columns described by these authors for the separation of the basic amino acids. Substitution of Dowex 50 × 4 resin for this purpose gave satisfactory results. Well-defined zones were obtained for the acidic and neutral amino acids on the 100 cm. column of Dowex 50 × 8.

Preparation of peroxide-oxidized corticotropin A_1

Corticotropin A_1 (9·8 mg.) was dissolved in water (1·5 ml.) and 0·05 N-sodium acetate solution (3·5 ml.) added. A 6% (w/v) H_2O_2 solution was added in three 0·05 ml. portions at 15 min. intervals. After a further 45 min. the reaction was stopped by the addition of 0·1 N-HCl (1·4 ml.). Excess of H_2O_2 was removed by shaking with about 20 mg. of platinum black, until a negative starch–potassium iodide reaction was obtained. The platinum was removed by centrifuging and 0·1 M-NaCl solution (1 ml.) was added to the supernatant liquid. Sodium ions were removed from the solution by passage through a short column containing Dowex 50 × 16 cation-exchange resin (0·25 g.) in the hydrogen form, Cl^- ions were exchanged for acetate with Amberlite IR-4 B anion-exchange resin in the acetate form (0·25 g.). The oxidized corticotropin was adsorbed from the combined filtrate and washings on to acid-washed oxycellulose (50 mg.) during a period of 20 hr. Elution was carried out with 0·1 N-HCl (3 × 2 ml.); Cl^- ions were exchanged for acetate with Amberlite IR-4 B acetate and the final eluate and washings freeze-dried. Yield, 7·0 mg.

Effect of pH *on the oxidation of corticotropin A_1 by hydrogen peroxide*

A volume (0·2 ml.) of a solution containing 1 mg. of corticotropin A_1/ml. was mixed with 0·6 ml. of the appropriate buffer (ionic strength $I = 0{\cdot}084$) and 0·2 ml. of 0·2 M-H_2O_2 added. After 30 min. at 25°, 1 ml. of 2 N-acetic acid was added and the excess of H_2O_2 destroyed by the addition of a few milligrams of platinum black. After centrifuging to remove the platinum, the supernatant liquid was separated and a portion taken for bioassay. A volume (1 ml.) was treated with cysteine hydrochloride (20 mg.) and the mixture maintained at 78° for 18 hr. After cooling, the solution was diluted for bioassay (Table 2, Expt. 2). The buffers used were (*a*) acetic acid–sodium acetate, pH 5·0; (*b*) primary–secondary phosphate, pH 6·0, 7·0 and 8·0; (*c*) $NaHCO_3$–Na_2CO_3, pH 8·5.

Table 2. *Oxidation of corticotropin*

EDTA, ethylenediaminetetra-acetic acid; OC, oxycellulose concentrate; A_1, corticotropin A_1.

Expt. no.	Initial potency of corticotropin preparation (i.u./mg.)	Oxidizing conditions	Oxidized potency (% of initial)	Reduced potency (% of initial)
1	A_1 92 (72–116)	H_2O_2; pH 7; 45 min.	11 (5–20)	108 (72–165)
2	A_1 116 (75–179)	0·04 M-H_2O_2; 30 min.;		
		pH 5·0 (acetate)	33 (17–61)	110 (72–181)
		pH 6·0 (phosphate)	22 (15–30)	108 (81–138)
		pH 7·0 (phosphate)	26 (13–50)	81 (73–90)
		pH 8·0 (phosphate)	17 (10–25)	78 (46–129)
		pH 8·5 (bicarbonate)	9 (7–15)	64 (30–144)
3	A_1 116 (75–179)	0·04 M-$K_2S_2O_8$; 30 min.; pH 3·0; 25°	<4	11 (8–18)
4	A_1 98 (62–154)	0·002 M-$K_3Fe(CN)_6$; 60 min.; pH 7·0 (acetate)	19 (12–27)	94 (49–178)
5	A_1 116 (75–179)	0·01 M-KIO_4; 8 min.; pH 9·2	7 (5–11)	10 (7–13)
6	OC 49 (39–64)	EDTA–$FeSO_4$–ascorbic acid–O_2; 120 min.; pH 5·5	<35	<35
7	A_1 116 (75–179)	Photo-oxidation, methylene blue; pH 8·3; irradiated for		
		10 min.	67 (42–106)	—
		30 min.	16 (13–21)	—
		60 min.	<2	<2

Oxidation of corticotropin A_1

Potassium peroxydisulphate. Corticotropin A_1 (0·54 mg.) in 0·1 N-acetic acid (0·5 ml.) was treated with 0·08 M-$K_2S_2O_8$ in 0·1 N-acetic acid (0·5 ml.) and the mixture kept at room temperature for 30 min. Peroxydisulphate was removed by passage through a short column of Dowex 1 × 8 anion-exchange resin in the acetate form. Comparison of the ultraviolet absorption of the filtrate at 265 and 275 mμ indicated that the specific tyrosine–tryptophan absorption band had disappeared. A volume (1 ml.) of the filtrate was treated with cysteine hydrochloride (20 mg.) at 78° for 18 hr. and diluted for bioassay (Table 2, Expt. 3).

Potassium ferricyanide. Corticotropin A_1 (0·47 mg.) was dissolved in 0·05 M-sodium acetate solution (1 ml.) and the solution adjusted to pH 7·0. Potassium ferricyanide solution (0·04 M, 0·05 ml.) was added and the mixture kept at room temperature for 1 hr. The reaction was terminated by the addition of 2 N-acetic acid (1 ml.), and $Fe(CN)_6^{3+}$ ions and any $Fe(CN)_6^{4+}$ ions formed were removed by passage through a short column of Dowex 2 × 8 anion-exchange resin in the acetate form. Portions of the oxidized solution, and of a portion reduced with cysteine hydrochloride (20 mg.) at 78° for 18 hr., were diluted for bioassay (Table 2, Expt. 4).

Potassium periodate (cf. Dixon, 1956). Corticotropin A_1 (8·4 mg.) and histidine hydrochloride (4·64 mg.) were dissolved in water (2·1 ml.) and the pH of the mixture was brought to 9·2 by the addition of 0·2 M-$K_2B_4O_7$ solution (3·5 ml.). Potassium periodate (0·1 M) in 0·2 N-KOH (0·625 ml.) was then added. After 8 min. KBH_4 (16 mg.) was added with continuous stirring. Three minutes later acetic acid (0·2 ml.) was added dropwise. The mixture was stirred for 30 min., M-NaCl (0·7 ml.) was then added and Na^+ ions were removed on a 5·1 cm. × 0·7 cm. column of Dowex 50 × 16 cation-exchange resin. Anions present in the effluent were exchanged for acetate by passage through a 10 cm. × 0·7 cm. column of Dowex 2 × 8 acetate. The combined effluent and washings were stirred for 20 hr. with oxycellulose (50 mg.) which had been previously washed with 0·1 N-acetic acid. The oxidized corticotropin was eluted from the oxycellulose with 0·1 N-HCl (2 ml.) followed by two washings (1 ml. each). The combined eluate and washings were freed from Cl^- ions by exchange with acetate from Dowex 2 × 8 exchange resin and freeze-dried. Yield, 5·2 mg.; potency, 8 i.u./mg. (Table 2, Expt. 5).

Ascorbic acid, ferrous sulphate and oxygen (cf. Udenfriend, Clark, Axelrod & Brodie, 1954). Oxycellulose-purified corticotropin (1 mg.) was treated with 0·1 ml. of a solution containing ascorbic acid (25 mg.), $FeSO_4$ (42 mg.) and ethylenediaminetetra-acetic acid (25 mg.) in water (3 ml.), and the pH adjusted to 5·5 with Na_2HPO_4 solution. The mixture was shaken in contact with air for 2 hr. and the reaction terminated by the addition of 0·1 N-acetic acid (1 ml.). All anions present in the solution were exchanged for acetate in Amberlite IRA-400 acetate. Portions of the eluate were taken for direct bioassay and for bioassay after reduction with cysteine hydrochloride (20 mg.) at 78° for 18 hr. (Table 2, Expt. 6).

Photochemical oxidation (cf. Weil, Gordon & Buchert, 1951). Corticotropin A_1 (2 mg.) was dissolved in water (1·9 ml.) containing $NaHCO_3$ (2·5 mg.) and methylene blue (0·1 mg.). The tube containing the mixture was cooled by a continuous flow of tap water. Irradiation was carried out with a bank of three 100 w electric-light lamps, the centre lamp being placed 15 cm. from the sample tube. Samples (0·6 ml. each) were withdrawn at 10, 30 and 60 min. Hydrochloric acid (N, 0·035 ml.) and Dowex 50 × 16 (H form; 50 mg.) were added to each sample, which was shaken mechanically for 15 min. This procedure removed Na^+ ions and methylene blue. The ion-exchange resin was removed by centrifuging, and a portion (0·05 ml.) of the supernatant liquid diluted for bioassay. The biological potencies of the products are given in Table 2 (Expt. 7).

A volume (0·5 ml.) of the remainder of each sample was evaporated to dryness *in vacuo* and hydrolysed by heating with 6 N-HCl (0·2 ml.) in a sealed capillary at 110° for 18 hr. The hydrolysates were freed from most of the HCl by repeated evaporation with water *in vacuo*. The samples, together with a hydrolysate of corticotropin A_1 prepared similarly, were subjected to paper electrophoresis in phosphate buffer (0·066 M, pH 5·9) at a potential gradient of 2·4 v/cm. for 17 hr. The positions of the histidine zones were determined on a reference strip by development with the diazotized *p*-anisidine reagent of Sanger & Tuppy (1951). The corresponding areas for the hydrolysates were cut out and the histidine was eluted with 0·1 N-HCl (2 ml.). The histidine contents were determined by the colorimetric procedure of MacPherson (1942), light absorption being measured at 530 mμ in 2 cm. cells. This procedure provides a convenient method for the determination of histidine in protein hydrolysates in the range of 2–10 μg. The amounts found were: original material, 3·3 % of histidine; 10 min. oxidation, 0·7 %; 30 min., 0·6 %; 60 min., 0·5 %.

Reduction of peroxide-oxidized corticotropin A_1

Thiol compounds at 78°. Oxidized corticotropin A_1 (0·23 mg.) was dissolved in 2 N-acetic acid (0·92 ml.). A volume (0·2 ml.) of this solution was diluted for direct bioassay. (*a*) 0·2 ml. was diluted to 1 ml. with water, cysteine hydrochloride (20 mg.) was

added and the mixture maintained at 78° for 18 hr. The solution was then diluted for bioassay. (*b*) 0·2 ml. was mixed with 0·8 ml. of a solution of cysteine acetate prepared by anion exchange from a solution of cysteine hydrochloride (25 mg./ml.). The mixture was kept at 78° for 18 hr. and diluted for bioassay. (*c*) 0·2 ml. was mixed with thioglycollic acid (0·02 ml., 26·5 mg.), diluted to 1 ml. and the pH adjusted to 2·5. The mixture was heated at 78° for 18 hr. and diluted for bioassay (Table 3, Expt. 8).

Other reducing agents at 37°. Oxycellulose-purified corticotropin (2·5 mg.) in 0·004 N-acetic acid (2·5 ml.) was oxidized with H_2O_2 and the solution freed from excess of H_2O_2 as described above. (*a*) 0·5 ml. was diluted to 1 ml. and the pH adjusted to 6·0. 2:3-Dimercaptopropanol (0·02 ml.) was added and the mixture kept at 37°. Samples for bioassay were removed at 41 and 90 hr. (*b*) 0·5 ml. was mixed with thioglycollic acid (0·02 ml., 15 mg.) and water (0·5 ml.). The mixture was kept at 37° and samples were removed at 41 and 90 hr. (*c*) 0·5 ml. was diluted to 1 ml. and cysteine hydrochloride (20 mg.) added. Portions were taken for bioassay after incubation for 41 and 90 hr. at 37°. (*d*) 0·5 ml. was diluted to 1 ml. and ascorbic acid (20 mg.) added. The mixture was kept at 37°, samples for bioassay being taken at 41 and 90 hr. Assay results are given in Table 3 (Expt. 9).

Potassium borohydride. Peroxide-oxidized corticotropin (OC purified; 2·2 mg.) was dissolved in water (2 ml.) and KBH_4 (20 mg.) added. The mixture was kept at 37°, portions for bioassay being taken at 30, 60 and 120 min. These were acidified with acetic acid before dilution (Table 3, Expt. 10).

Methionine. Corticotropin A_1 (1 mg.) was dissolved in 0·1 M-$NaHCO_3$ and oxidized by the addition of 6% H_2O_2 (3 × 0·01 ml.). The reaction was terminated by the addition of 2 N-acetic acid (1 ml.) and excess of H_2O_2 decomposed by shaking with platinum black. After separation from the platinum a portion was taken for direct bioassay. A volume (1 ml.) of the solution was treated with methionine (20 mg.) and maintained at 75° for 18 hr. A portion of the mixture was diluted for bioassay (Table 3, Expt. 11).

Treatment of corticotropin A_1 with sodium p-*chloromercuribenzoate*

Corticotropin A_1 (0·22 mg.) was dissolved in water (1 ml.) and sodium *p*-chloromercuribenzoate (0·5 mg.) add[illegible]. The mixture was shaken mechanically for 1 hr. and diluted to 4·4 ml. with 0·1 N-acetic acid. This solution was further diluted for bioassay (Table 4, Expt. 12*a*).

Oxidation–reduction of corticotropin A_1 after treatment with sodium p-*chloromercuribenzoate*

Corticotropin A_1 (0·44 mg.) was dissolved in 0·02 M-phosphate buffer, pH 7·5 (2·2 ml.) and 0·2 ml. was withdrawn for bioassay. The remainder of the solution was shaken with sodium *p*-chloromercuribenzoate (0·2 mg.) for 10 min. Oxidation was carried out as described earlier with 3 × 0·02 ml. of 6% (w/v) H_2O_2 for 45 min., the reaction being terminated by the addition of 0·1 N-acetic acid (2 ml.). Excess of H_2O_2 was decomposed with platinum black and, after centrifuging, the solution was passed through a 1 cm. × 0·7 cm. column of Dowex 2 × 8 acetate. The filtrate and column washings were diluted to 8 ml. with water. A portion of this solution was taken for direct bioassay, 2 ml. was treated with cysteine hydrochloride (40 mg.) for 18 hr. at 78° and finally diluted for bioassay. The results are given in Table 4 (Expt. 12*b*).

Raney-nickel desulphurization of corticotropin A_1

Corticotropin A_1 (2·2 mg.) was dissolved in a mixture of 0·1 N-acetic acid (0·4 ml.) and ethylene glycol monomethyl ether (purified by distillation

Table 3. *Reduction of oxidized corticotropin*

OC, Oxycellulose concentrate; A_1, corticotropin A_1.

Expt. no.	Initial potency of corticotropin preparation (i.u./mg.)	Potency after oxidation (% of initial)	Reducing conditions	Reduced potency (% of initial)
8	A_1 116 (75–179)	24 (16–39)	(*a*) 2% Cysteine hydrochloride (18 hr.; 75°)	75 (47–132)
			(*b*) 2% Cysteine acetate (18 hr.; 75°)	108 (72–165)
			(*c*) 2% Thioglycollic acid (buffered to pH 2·5; 18 hr.; 75°)	112 (47–182)
9	OC 53 (33–85)	11 (5–20)	(*a*) 2% 2:3-Dimercaptopropanol (pH 6·0; 90 hr.; 37°)	87 (68–110)
			(*b*) 2% Thioglycollic acid (90 hr.; 37°)	70 (55–87)
			(*c*) 2% Cysteine hydrochloride (90 hr.; 37°)	102 (49–188)
			(*d*) 2% Ascorbic acid (90 hr.; 37°)	30 (26–34)
10	A_1 116 (75–179)	24 (16–39)	1% KBH_4 (30 min.; 25°)	26 (18–49)
11	A_1 120 84–205)	13 (7–23)	2% Methionine (pH 3·0; 18 hrs; 75°)	27 (21–36)

from $SnCl_2$; 2 ml.). Raney nickel (approx. 50 mg.) was added and the tube shaken to give a uniform suspension. Air was displaced from the tube and solution by repeated evacuation and admission of H_2. The stoppered tube was maintained at 87–90° for 7 hr. with continuous agitation. The mixture was then cooled, the nickel separated by centrifuging, and the supernatant liquid evaporated *in vacuo* to a volume of approximately 0·2 ml. The Raney nickel was washed three times with 2 ml. portions of 0·01 N-acetic acid by vigorous shaking for 3–5 min. The washings were combined with the concentrated supernatant liquid and a freshly prepared solution of cysteine acetate was added to bring the solution to a concentration of 20 mg./ml. The mixture was maintained at 78° for 18 hr. and the corticotropin adsorbed on to acid-washed oxycellulose (10 mg.). Corticotropin was eluted from the oxycellulose with 0·1 N-HCl (2 ml.), Cl^- ions were exchanged for acetate with Dowex 2 × 8 acetate, and the solution was freeze-dried. Yield, 1·5 mg. The methionine content of the desulphurized and original materials were determined on HCl hydrolysates by quantitative paper chromatography. The values obtained were: corticotropin A_1 (preparation 43/3) methionine, 2·8%; Raney-nickel-treated corticotropin A_1 (43/3), less than 0·5%.

Oxidation and reduction of corticotropin A_1 treated with Raney nickel

Raney-nickel-treated corticotropin A_1 (0·09 mg.; methionine content less than 0·7%) was dissolved in 0·01 M-$NaHCO_3$ (0·4 ml.). Oxidation was carried

Table 4. *Reactions of corticotropins*

OC, Oxycellulose concentrate; A_1, corticotropin, A_1.

Expt. no.	Initial potency of corticotropin preparation (i.u./mg.)	Reaction	Potency (% of initial)	Reduced potency (% of initial)
12*a*	A_1 120 (84–176)	*p*-Chloromercuribenzoate (60 min.; 25°)	87 (57–133)	—
12*b*	A_1 72 (50–103)	*p*-Chloromercuribenzoate (60 min.; 25°; oxidized H_2O_2)	38 (24–63)	120 (100–141)
13*a*	A_1 81 (78–85)	Raney nickel (7 hr.; 90°)	89 (42–185)	—
13*b*	A_1 Desulphurized as above	Oxidized H_2O_2	<12	99 (53–182)
14*a*	A_1 116 (75–179)	Dimedon (pH 4·6; 3 hr.; 25°)	121 (70–210)	—
14*b*	Oxidized A_1 26 (10–68)	Dimedon (pH 4·6; 3 hr.; 25°)	—	115 (85–164)
15*a*	OC 42 (28–62)	2:4-Dinitrophenylhydrazine in 99% acetic acid (10 min.; 70°)	52 (36–76)	—
15*b*	Oxidized OC 12 (8–19)	2:4-Dinitrophenylhydrazine in 99% acetic acid (10 min.; 70°)	—	81 (62–110)
16*a*	OC 42 (28–62)	1% Thiosemicarbazide in 0·1 N-acetic acid (6 hr.; 25°)	100 (41–226)	—
16*b*	Oxidized OC 12 (8–19)	1% Thiosemicarbazide in 0·1 N-acetic acid (6 hr.; 25°)	—	95 (64–131)
17*a*	A_1 116 (75–179)	0·1 N-NaOH (2 min.; 25°)	122 (72–216)	—
17*b*	Oxidized A_1 14 (8–30)	0·1 N-NaOH (2 min.; 25°)	—	113 (65–194)
18*a*	A_1 92 (73–115)	—	—	—
18*b*	Oxidized A_1 10 (5–17)	NaCN (pH 3·0; 30 min.)	—	112 (71–178) of 18*a*
19*a*	A_1 92 (73–115)	I_2 (pH 6·5; 30 min.)	22 (21–24)	—
19*b*	Iodinated A_1	H_2O_2 (pH 7·0)	5 (6–7)	58 (51–64)
20*a*	OC 32 (22–49)	5 N-HCl (25 min.; 25°)	81 (47–141)	—
20*b*	OC 32 (22–49)	4% Dimethylaminobenzaldehyde in 5 N-HCl (25 min.; 25°)	<10	—
20*c*	Oxidized OC 10 (8–14)	4% Dimethylaminobenzaldehyde in 5 N-HCl (25 min.; 25°)	—	<10
21*a*	A_1 116 (75–179)	—	—	—
21*b*	Oxidized A_1 40 (29–55)	0·03 N-HCl (18 hr.; 78°)	25 (20–48) of 21*a*	90 (62–126) of 21*a*

out with 6 % (w/v) H_2O_2 (3 × 0·01 ml.) during 45 min. The reaction was terminated by the addition of 2 M-acetic acid (1·4 ml.) and excess of H_2O_2 destroyed by shaking with platinum black. After removal of the platinum by centrifuging, a portion of the solution was removed for direct assay, and 1 ml. was reduced with cysteine hydrochloride (20 mg.) at 78° for 18 hr. before bioassay. The results of this experiment, together with the bioassay of the desulphurized corticotropin A_1, are given in Table 4, Expt. 13*a* and *b*.

Effect of various reagents on corticotropin A_1 and peroxide-oxidized corticotropin

Dimedone. Solutions of corticotropin A_1 (0·26 mg.) and oxidized corticotropin A_1 (0·25 mg.) were prepared in 0·05 M-acetate buffer, pH 4·6 (0·9 ml.). A 1 % (w/v) ethanolic solution of dimedone (5:5-dimethyl*cyclo*hexane-1:3-dione; 0·1 ml.) was added to each solution, and the mixtures were kept at room temperature for 3 hr. Acetic acid (0·5 N, 1 ml.) was then added to each. A volume (1 ml.) of the oxidized corticotropin solution was reduced with cysteine hydrochloride (20 mg.) at 78° for 18 hr. Portions of both solutions were diluted for bioassay (Table 4, Expt. 14).

2:4-*Dinitrophenylhydrazine.* Oxycellulose-purified corticotropin (0·5 mg.) and a peroxide-oxidized corticotropin made from the same preparation (0·5 mg.) were each dissolved in a saturated solution of 2:4-dinitrophenylhydrazine in 99 % (v/v) acetic acid (0·1 ml.). The mixtures were kept at 70° for 10 min. and diluted with water to 1 ml. Excess of 2:4-dinitrophenylhydrazine was removed from each solution by passage through 3·5 cm. × 0·7 cm. columns of Amberlite IR-100 cation-exchange resin (H form) and the columns were washed with 0·1 N-acetic acid (3 ml.). Eluates and washings from each column were combined. A volume (1 ml.) of the oxidized corticotropin solution was reduced with cysteine hydrochloride (20 mg.) at 78° for 18 hr. before bioassay (Table 4, Expt. 15).

Thiosemicarbazide. Oxycellulose-purified corticotropin (1 mg.) and a corresponding oxidized preparation (1 mg.) were dissolved in 0·1 N-acetic acid (1 ml.) and thiosemicarbazide (10 mg.) was added to each solution. The mixtures were kept for 6 hr. at room temperature, excess of thiosemicarbazide was removed by means of short columns of Amberlite IR-100 cation-exchange resin (H form; 0·5 g.) and the columns were washed with 0·05 N-HCl (2 ml.). Both solutions were diluted with water to 6·3 ml. and the oxidized corticotropin solution was reduced with cysteine hydrochloride (20 mg.) at 78° for 18 hr. before bioassay (Table 4, Expt. 16).

Alkali. Corticotropin A_1 (0·33 mg.) and the corresponding peroxide-oxidized corticotropin A_1 (0·3 mg.) were dissolved separately in water (1·4 ml. portions), and N-NaOH (0·1 ml.) was added to each solution. The ultraviolet absorption at 275 mμ was measured 30, 60 and 120 sec. after the addition of alkali. Acetic acid (2 N, 0·5 ml.) was added to both solutions after 2 min. in order to terminate the reaction. Portions of each solution were reduced with cysteine hydrochloride (20 mg.) at 78° for 18 hr. and diluted for bioassay (Table 4, Expt. 17). No change in the optical density at 275 mμ was observed during the experimental period with either solution.

Acid. Peroxide-oxidized corticotropin A_1 (0·39 mg.) in 0·03 N-HCl (2 ml.) was heated at 78° for 18 hr. After cooling, 1 ml. was diluted for direct bioassay and the remainder was reduced with cysteine hydrochloride (20 mg.) at 78° for 18 hr. before dilution for bioassay (Table 4, Expt. 21).

Cyanide. Peroxide-oxidized corticotropin A_1 (0·53 mg.) in 0·1 N-acetic acid (1·5 ml.) was treated with NaCN (1 mg.). After 30 min. CN^- ions were removed by exchange on Dowex 2 × 8 acetate (approx. 10 mg.). A portion of the solution was diluted for direct bioassay and a further 1 ml. portion reduced with cysteine hydrochloride (20 mg.) at 78° for 18 hr. A portion of the oxidized corticotropin A_1 was reduced under identical conditions for comparison (Table 4, Expt. 18).

p-*Dimethylaminobenzaldehyde.* Oxycellulose-purified corticotropin (2 mg.) and a corresponding peroxide-oxidized preparation (2 mg.) were each dissolved in 5 N-HCl (0·05 ml.) and a 4 % (w/v) solution of *p*-dimethylaminobenzaldehyde in 5 N-HCl (0·05 ml.) was added to each solution. The mixtures were kept at room temperature for 25 min. and water (1·4 ml.) was finally added to each. The absorption spectra of both solutions were measured at wavelengths from 530 to 640 mμ at 10 mμ intervals. No qualitative or quantitative differences were observed in the spectra (absorption maxima at 540 and 580 mμ). Portions of each reaction mixture were diluted for bioassay. A portion of the oxidized corticotropin reaction mixture was reduced with cysteine hydrochloride (20 mg./ml.) at 78° for 18 hr. and diluted for bioassay. A control experiment was carried out with oxycellulose-purified corticotropin (2 mg.) in 5 N-HCl (0·05 ml.). This solution was kept at room temperature for 25 min., neutralized and diluted for bioassay (Table 4, Expt. 20).

Iodination and oxidation of corticotropin A_1

Corticotropin A_1 (1·1 mg.) in 0·05 M-acetate buffer, pH 6·5 (0·7 ml.), was treated with 0·01 N-I_2 solution (0·2 ml.), equivalent to 6 atoms of I/tyrosine residue, calculated on a tyrosine content of 6·2 %. The mixture was kept at room temperature for 30 min. Measurement of the ultraviolet absorption at 312 mμ indicated that 81 % of the

tyrosine was converted into 3:5-di-iodotyrosine. During the reaction period a small amount of precipitate formed. This was redissolved finally by the addition of 2N-acetic acid (0·5 ml.) to the mixture. Sodium thiosulphate (0·01N, 0·2 ml.) was then added to remove excess of I_2 and anions in the mixture were exchanged for acetate by passage of the solution through a short column of Dowex 2 × 8 acetate. Eluate and column washings amounted to 2·9 ml.; 0·9 ml. of this solution was diluted for direct bioassay. Another portion (1 ml.) was adjusted to pH 7·0 and oxidized with 6% (w/v) H_2O_2 (3 × 0·01 ml.). Excess of H_2O_2 was removed by shaking with platinum black. A portion of the separated supernatant liquid was diluted for bioassay, and a further portion reduced with cysteine hydrochloride (20 mg./ml.) at 75° for 18 hr. before bioassay (Table 4, Expt. 19). Examination of the ultraviolet absorption of this solution at 312 mμ indicated that the 3:5-di-iodotyrosine content was unchanged.

DISCUSSION

The evidence presented in the Experimental section makes it clear that the adrenal ascorbic acid-depleting potency of corticotropin preparations can be destroyed by oxidation with hydrogen peroxide or ferricyanide and that the biological activity of such materials can be quantitatively restored by treatment with thiols. Up to the present time, no reducing agent other than a thiol compound has been found to be effective in this reduction. Ascorbic acid, potassium borohydride, sodium thiosulphate, hydrogen and platinum, zinc and hydrochloric acid and electrolytic reduction are equally ineffective in restoring the biological activity of peroxide-oxidized corticotropin. Since the publication of our preliminary communication, the peroxide oxidation and thiol reduction of corticotropin have been confirmed by Dixon & Stack-Dunne (1955), who have also demonstrated differences in the chromatographic behaviour of the untreated and oxidized forms (see also Dixon, 1955, 1956). Farmer & Morris (1956) have also been able to separate oxidized from unchanged corticotropin A_1 by chromatography on a column of carboxymethylcellulose. There is therefore no doubt that the oxidized form of corticotropin represents a chemically distinct species. The oxidation process appears to be pH dependent (Table 2), being more effective at alkaline than at acid pH values. On the alkaline side of neutrality, however, some irreversible oxidation may occur. On the other hand, reduction with thiols is more rapid at low pH values, although the pH effect is less marked than with the oxidation process.

In contrast to the action of hydrogen peroxide and ferricyanide, peroxydisulphate and periodate bring about irreversible inactivation. The periodate inactivation may be explained by the oxidation of the *N*-terminal serine residue in corticotropin, which other studies (White, 1955) have shown to be essential for the biological activity of the hormone.

The chemical nature of the oxidation–reduction centre in corticotropin is not readily explained by present knowledge of the structure of the hormone. Bell (1954) and Howard, Shepherd, Eigner, Davies & Bell (1955) determined the amino acid sequence of β-corticotropin, a highly purified and apparently homogeneous preparation obtained by countercurrent distribution of oxycellulose concentrates of pig corticotropin. The structure proposed by these workers is a single chain of thirty-nine amino acid residues. Part of this chain may be removed by the action of pepsin without reduction in biological activity. The essential sequence is: Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro.Val.Gly.Lys.Lys.Arg.Arg.Pro.Val.Lys.Val.Tyr.Pro.Asp.Gly.Ala.Glu. [For definitions of these abbreviations see *Biochem. J.* (1953), **55**, 5.]

The amino acid composition of our preparation of corticotropin A_1 given in Table 5 is consistent with the structure proposed by Bell (1954) and differs only in a somewhat higher proline content.

Countercurrent studies kindly carried out by Dr P. H. Bell, with a similar preparation, showed that it contained 67% of β-corticotropin.

It may be concluded that the structure proposed for β-corticotropin by Howard *et al.* (1955) is also

Table 5. *Amino acid composition of corticotropin A_1 and of peroxide-oxidized corticotropin A_1*

	Corticotropin A_1		Oxidized corticotropin A_1	
Amino acid	Concn. (g./100 g.)	Minimum no. of residues	Concn. (g./100 g.)	Minimum no. of residues
Alanine	4·46	2·9	4·84	3·0
Arginine	8·66	2·9	9·41	3·0
Aspartic acid	5·57	2·4	4·97	2·0
Glutamic acid	13·34	5·3	14·07	5·2
Glycine	4·20	3·3	4·13	3·0
Histidine	2·94	1·1	3·06	1·1
Leucine	4·50	2·0	5·10	2·1
Lysine	14·07	5·6	13·49	5·0
Methionine	2·40	0·9	2·17	0·8
Phenylalanine	8·88	3·1	8·52	2·8
Proline	12·22	6·2	10·48	5·2
Serine*	3·30	1·8	3·06	1·6
Tryptophan†	4·17	1·2	3·65	1·0
Tyrosine	6·22	2·0	6·52	2·0
Valine	5·32	2·6	5·33	2·5

* Corrected for destruction on hydrolysis.
† Calculated from ultraviolet absorption (Beaven & Holiday, 1952).

valid for the preparations of corticotropin A_1 used in the present studies.

Only five of the amino acids present in the essential sequence could conceivably be involved in a reversible oxidation–reduction system: serine, tyrosine, methionine, histidine and tryptophan. The presence in the hormone molecule of a sulphydryl–disulphide system which has hitherto escaped detection is excluded by the failure of *p*-chloromercuribenzoate to prevent oxidation and subsequent reduction (Table 4, Expt. 12), and by the fact that desulphurization with Raney nickel under very vigorous conditions also does not affect the oxidation–reduction process (Table 4, Expt. 13). The latter finding also excludes methionine, as the analytical results show that the single residue of this amino acid in the hormone molecule can be completely desulphurized without change in the biological potency of corticotropin A_1 and without impairing the ability to undergo reversible oxidation–reduction. It is interesting that the methionine contents of untreated and oxidized hormones given in Table 5 indicate that this amino acid is not converted into the sulphone or sulphoxide during the oxidation process.

Expts. 14, 15, 16 and 18 (Table 4) were designed to test the possibility that a carbonyl compound was formed on oxidation. A somewhat unlikely possibility was that the primary alcoholic group of the *N*-terminal serine residue might be oxidized to an aldehyde group either directly or through a hydroperoxide. If a carbonyl group were present in the oxidized form, reaction with carbonyl reagents such as dimedone, 2:4-dinitrophenylhydrazine or thiosemicarbazide should prevent subsequent reduction with restoration of biological activity. Expts. 14, 16 and 18 with dimedone, thiosemicarbazide and cyanide ions show that this blocking does not occur. Expt. 15 is less conclusive, owing to the inactivating effect of 2:4-dinitrophenylhydrazine on corticotropin itself, but does confirm the absence of the blocking effect. These experiments, together with the unchanged serine contents shown in Table 5, eliminate the possibility of this amino acid being involved in the oxidation centre.

It has never been demonstrated that histidine can take part in a reversible oxidation–reduction system, although Weil & Buchert (1951) have shown that it can be oxidized in intact proteins under relatively mild conditions. Photochemical oxidation at pH 8·3 in air, with methylene blue as oxygen carrier, resulted in a progressive irreversible inactivation, which was, however, not parallel to the decrease in the histidine content (Table 2, Expt. 7). The fall in histidine content to 22% of the original value after photo-oxidation for 10 min., without any significant alteration in biological activity, indicates that this amino acid, like methionine, is probably not essential for hormonal activity. This fact, together with the unchanged histidine content of the oxidized hormone, makes it very improbable although not impossible that histidine is involved in the oxidation–reduction centre. It is possible that histidine, although not essential for biological activity, may be modified in the oxidized form, but the chemical basis for this is not clear, especially as the modified form must be chromatographically indistinguishable from the parent amino acid. The irreversible inactivation after further photo-oxidation is probably due to destruction of the aromatic amino acids (Weil *et al.* 1951).

The oxidation of tyrosine in intact proteins or peptides has been demonstrated many times, although there is very little evidence that a reversible process can occur. In the present case, the stability of the oxidized form to 0·1 N-NaOH (Table 4, Expt. 17) makes it unlikely that the latter is a catechol derivative. This is confirmed by the apparent inability of the oxidized form to form an acidic boric acid complex. Hydroxylation by the method of Udenfriend *et al.* (1954), which specifically introduces hydroxyl groups into aromatic rings, results in irreversible inactivation of the hormone (Table 2, Expt. 6). This may, of course, be due to conversion of phenylalanine into tyrosine residues or hydroxylation of tryptophan in the hormone molecule.

Iodination of corticotropin A_1 (Table 4, Expt. 19) is more difficult to interpret. Iodination with 6 atoms of iodine/tyrosine residue at pH 6·5 gives a reaction product which is less active than the starting material. This can be oxidized with hydrogen peroxide to an almost inactive product, which can in turn be reduced to give a biologically active material which is intermediate in activity between the original and the iodinated hormone. Iodination thus does not appear to eliminate the oxidation–reduction centre, although it does lead to some irreversible inactivation.

The evidence of these experiments, together with the unaltered ultraviolet absorption spectra of the oxidized form under both acid and alkaline conditions (Fig. 2) and the unchanged tyrosine content (Table 5), make it very unlikely that tyrosine is involved in the oxidation–reduction centre of corticotropin.

The evidence for tryptophan is less conclusive. It is clear from the identity of the ultraviolet-absorption spectra of untreated and oxidized corticotropins that no extensive changes in the tryptophan molecule can occur. The tryptophan contents of the two preparations, estimated from the spectra by the method of Beaven & Holiday (1952), are very similar, although that of the oxidized preparation is somewhat lower.

Condensation of both untreated and oxidized preparations with *p*-dimethylaminobenzaldehyde in the presence of strong hydrochloric acid (Table 4, Expt. 20) leads to irreversible inactivation in both cases, indicating that the 2 position of the tryptophan residue is probably essential for biological activity, although the possibility of side reactions cannot be excluded. The identical absorption spectra of the coloured condensation products suggests that the 2 positions of the tryptophan residues are probably in similar states in both preparations and excludes a residue such as oxindolylalanine in the oxidized form. This conclusion is also supported by the ultraviolet-absorption spectra in alkaline solution (Cornforth, Dalgliesh & Neuberger, 1951).

The oxidation products of tryptophan have attracted some attention in recent years because of their importance in the first stages of the metabolism of this amino acid. A number of possible hydroxylated or peroxide derivatives have been suggested [reviewed by Ek, Kissman, Patrick & Witkop (1952) and by Dalgliesh (1955)] as the first products of the action of the enzyme tryptophan peroxidase on the amino acid. With the exception of oxindolylalanine none of these has been isolated or synthesized, so that their properties are unknown. The relatively high stability of oxidized corticotropin under acid (Table 4, Expt. 21) and alkaline conditions (Table 4, Expt. 17) makes its formulation as a hydroperoxide unlikely.

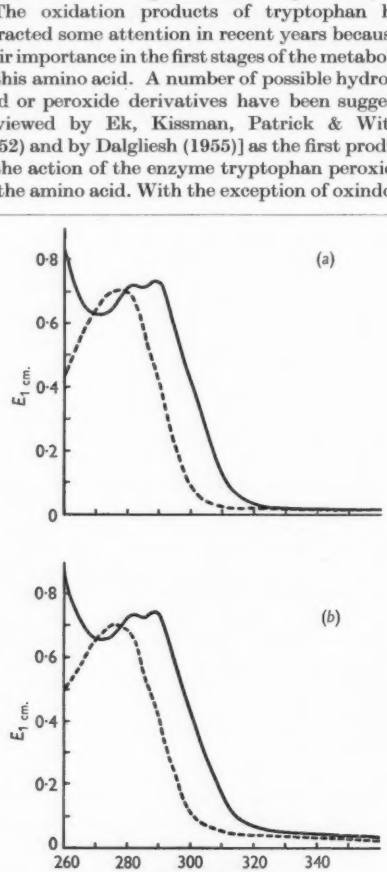


Fig. 2. Ultraviolet-absorption spectra of untreated (*a*) and peroxide-oxidized corticotropin (*b*). ——, 0·1 N-NaOH; - - - - -, 0·01 N-HCl.

Houff, Hinsvark, Weller, Wittiver & Sell (1954) have suggested that *N*-hydroxyindole derivatives are formed when certain indole compounds are oxidized with ferric chloride and perchloric acid. The indoles examined included several substituted carboxylic acids, but did not include tryptophan. Attempts to prepare similar N_1-hydroxy derivatives from tryptophan or acetyltryptophan in this laboratory have been unsuccessful. Houff *et al.* (1954) postulate a dimer as an intermediate in the formation of the *N*-hydroxyindoles, and it is possible that the formation of this compound is difficult with tryptophan or its peptides.

The experiments reported here, together with amino acid analyses presented in Table 5, make it improbable that any of these amino acids are concerned in the oxidation–reduction process. A possible exception is provided by histidine and by tryptophan, where the evidence is less conclusive and where a variety of oxidation products are theoretically possible.

Two alternatives remain: either the substance isolated as β-corticotropin is not in fact the biologically active hormone or the β-corticotropin molecule contains some hitherto unidentified group which determines the oxidation–reduction behaviour. The former alternative appears extremely unlikely in view of the impressive evidence of homogeneity presented by Bell (1954).

SUMMARY

1. The conditions for the oxidation of corticotropin to a biologically inactive product assayed by the adrenal ascorbic acid-depletion method, and for the reduction of this material with complete restoration of activity, have been investigated.

2. The nature of the groupings concerned in the oxidation–reduction process have been examined by studying the effect of various chemical treatments on this process.

3. Amino acid analyses of untreated and oxidized corticotropin A_1 are presented.

4. The chemical nature of the oxidation–reduction centre is discussed. It is concluded that, with the possible exception of tryptophan, the oxidation–reduction behaviour of the hormone cannot be accounted for by the amino acid residues known to be present in the corticotropin molecule.

The authors are indebted to Miss G. Atfield, who carried out the amino acid analyses by ion-exchange chromatography.

REFERENCES

Astwood, E. B., Raben, M. S., Payne, R. W. & Grady, A. B. (1951). *J. Amer. chem. Soc.* **73**, 2969.
Beaven, G. H. & Holiday, E. R. (1952). *Advanc. Protein Chem.* **7**, 375.
Bell, P. H. (1954). *J. Amer. chem. Soc.* **76**, 5565.
Cornforth, J. W., Dalgliesh, C. E. & Neuberger, A. (1951). *Biochem. J.* **48**, 598.
Cortis-Jones, B., Crooke, A. C., Henly, A. A., Morris, P. & Morris, C. J. O. R. (1950). *Biochem. J.* **46**, 173.
Dalgliesh, C. E. (1955). *Advanc. Protein Chem.* **10**, 33.
Dedman, M. L., Farmer, T. H. & Morris, C. J. O. R. (1955). *Biochem. J.* **59**, xii.
Dedman, M. L., Farmer, T. H., Morris, P. & Morris, C. J. O. R. (1952). *Recent Progr. Hormone Res.* **7**, 59.
Dixon, H. B. F. (1955). *Biochim. biophys. Acta*, **18**, 599.
Dixon, H. B. F. (1956). *Biochem. J.* **62**, 25P.
Dixon, H. B. F., Moore, S., Stack-Dunne, M. P. & Young, F. G. (1951). *Nature, Lond.*, **168**, 1044.
Dixon, H. B. F. & Stack-Dunne, M. P. (1955). *Biochem. J.* **61**, 483.
Ek, A., Kissman, A., Patrick, J. B. & Witkop, B. (1952). *Experientia*, **8**, 36.
Farmer, T. H. & Morris, C. J. O. R. (1956). *Nature, Lond.*, **178**, 1465.
Hirs, C. W. H., Moore, S. & Stein, W. H. (1953). *J. biol. Chem.* **200**, 493.
Houff, W. H., Hinsvark, O. N., Weller, L. E., Wittiver, S. H. & Sell, H. M. (1954). *J. Amer. chem. Soc.* **76**, 5654.
Howard, K. S., Shepherd, R. G., Eigner, E. A., Davies, D. S. & Bell, P. H. (1955). *J. Amer. chem. Soc.* **77**, 3419.
Kuehl, F. A., Meisinger, M. A. P., Brink, N. G. & Folkers, K. (1953). *J. Amer. chem. Soc.* **75**, 1955.
McFarren, E. F. (1951). *Analyt. Chem.* **23**, 168.
MacPherson, H. T. (1942). *Biochem. J.* **36**, 59.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
Morris, C. J. O. R. (1951). *Analyst*, **76**, 470.
Payne, R. W., Raben, M. S. & Astwood, E. B. (1950). *J. biol. Chem.* **187**, 719.
Pugsley, L. I. (1946). *Endocrinology*, **39**, 161.
Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 463.
Sayers, M. A., Sayers, G. & Woodbury, L. A. (1948). *Endocrinology*, **42**, 379.
Udenfriend, S., Clark, C. T., Axelrod, J. & Brodie, B. B. (1954). *J. biol. Chem.* **208**, 731.
Wael, J. de & Cadavieco, R. D. (1954). *Rec. Trav. chim. Pays-Bas*, **73**, 333.
Weil, L. & Buchert, A. R. (1951). *Arch. Biochem. Biophys.* **34**, 1.
Weil, L., Gordon, W. G. & Buchert, A. R. (1951). *Arch. Biochem. Biophys.* **33**, 90.
White, W. F. (1955). *J. Amer. chem. Soc.* **77**, 4691.
White, W. F. & Fierce, W. L. (1953). *J. Amer. chem. Soc.* **75**, 245.

The Estimation of Serum Protein-Bound Iodine by Alkaline Incineration

By J. D. ACLAND

Department of Pharmacology and Therapeutics, University of Sheffield, and Sheffield Royal Infirmary

(*Received* 14 *September* 1956)

The term 'protein-bound iodine' (PBI) is used to describe that fraction of blood iodine which is non-dialysable and precipitates with serum or plasma proteins (Trevorrow, 1939). In the absence of structural damage to, or carcinoma of, the thyroid, about 75–100% of serum PBI consists of L-thyroxine bound to protein, in a way as yet undefined, from which it can be released by denaturation. About 90% of the thyroxine is selectively combined with a 'thyroxine-binding-protein' fraction, intermediate in properties between α_1- and α_2-globulins, while the remainder is associated with albumin (Taurog & Chaikoff, 1948; Gordon, Gross, O'Connor & Pitt-Rivers, 1952; Petermann, Robbins & Hamilton, 1954; Robbins, Petermann & Rall, 1955). Of the other physiologically active compounds secreted by the thyroid, 3:5:3′-tri-iodothyronine is bound to protein in a similar way to thyroxine, though less strongly (Deiss, Albright & Larson, 1953), whereas no data are available for 3:3′-di-iodothyronine or for 3:3′:5′-tri-iodothyronine (Roche, Michel, Wolf & Nunez, 1956).

Salter & McKay (1944) were the first workers to describe a method of estimating serum PBI, in which organic material was destroyed by alkaline incineration. Liberated iodide was then determined, after elution, by its catalytic effect on the reduction of ceric salts by arsenious acid (Sandell & Kolthoff, 1937). This technique was subsequently modified by Barker & co-workers (Barker & Humphrey, 1950; Barker, Humphrey & Soley, 1951). Brown, Reingold & Samson (1953) and Grossmann & Grossmann (1955) described further modifications, which were claimed to increase the accuracy of the method. Doubt was cast by some of these authors on certain aspects of the technique, different workers not being always in agreement about the efficiency of a particular step.

The present investigation was started with the dual object of resolving the contradictions between

the findings of previous workers and of developing from the latter a reasonably reliable method. A re-investigation of all the stages of the analysis was undertaken. The results obtained with the zinc hydroxide method of precipitating PBI were compared with those observed when trichloroacetic acid precipitation was used. The effect of increasing the ashing time from $2\frac{1}{2}$ to $3\frac{1}{2}$ hr. and that of increasing the ashing temperature range from 590–610° to 640–660° were investigated and the iodide-elution procedures described by the above authors were compared. The kinetics of the reaction between ceric and arsenite ions were re-investigated from the point of view of its analytical application, and interference with this reaction by zinc salts was studied. The percentage recovery of DL-thyroxine added to serum was determined. Preliminary reports have appeared (Acland, 1955, 1956).

EXPERIMENTAL

Determination of serum PBI

The complete method finally adopted for serum is described in this section. Variants tested are dealt with later. No elementary iodine is allowed to be introduced into the room in which analyses are carried out.

Glassware. Before being used, all glass apparatus was kept immersed, at least overnight, in an aqueous solution of Dymex (J. W. Towers and Co. Ltd.), an alkaline detergent, to remove traces of adsorbed iodide. Immediately before use, the glassware was first rinsed thoroughly with tap water, test tubes being cleaned with a brush, and then washed three times with water purified as described below. Pipettes were dried with acetone on a filter pump, other glassware in a hot-air oven at 90°.

Reagents. All chemicals were AnalaR grade except HCl, H_2SO_4 ('Microanalytical reagent,' Hopkin and Williams Ltd.) and ceric ammonium sulphate ('Laboratory reagent low in other rare earths', British Drug Houses Ltd.). All water was purified by distillation from a metal still and passage through two columns of Amberlite Monobed MB-1 ion-exchange resin. The second resin treatment was given immediately before use. Distillation from permanganate or from alkali was not tried as satisfactory results were obtained with water purified in this way.

The KI reference solution contained 130·8 mg. of the desiccator-dried salt/l. ($\equiv 100\,\mu$g. of I^-/ml.). The ceric ammonium sulphate solution contained 39 g. of salt in 1 l. of 3·5 N-H_2SO_4. Arsenious acid was prepared by dissolving 3·8 g. of As_2O_3 in 50 ml. of warm N-NaOH. To the cooled solution were added 50 ml. of water and 3·5 N-H_2SO_4 to 500 ml.

Precipitation of PBI. To 1 ml. of serum in a 16 mm. × 125 mm. Pyrex tube was added water (7 ml.), followed by 1 ml. of 10% (w/v) $ZnSO_4,7H_2O$ and 1 ml. of 0·5 N-NaOH, the contents of the tube being thoroughly stirred after each addition by means of a glass rod. The protein precipitate was allowed to stand for 3 hr. and then separated by centrifuging in an angle centrifuge at 2000 rev./min. for 10 min. The supernatant was decanted and the precipitate washed three times by resuspending it in successive 10 ml. portions of water, stirring with a glass rod, centrifuging and discarding the washings. A fine suspension of the precipitate in 1 ml. of 4 N-Na_2CO_3 was then prepared with the glass rod, from which traces of precipitate were washed into the tube by adding 0·5–1 ml. of water down the rod.

Incineration of protein precipitate. The precipitate was dried overnight at 95–98° in an electric oven. The tube containing the dried precipitate was then placed in a muffle furnace for $3\frac{1}{2}$ hr. at 600°, the furnace door being left slightly open (about $\frac{1}{4}$ in.) to create a draught. The Pyrex tubes were discarded after having been used three times, since they became badly etched and difficult to clean.

Estimation of released iodide. After the tube had cooled, 1 ml. of 2 N-HCl was added carefully to the residue and thoroughly mixed with a glass rod. Water (6 ml.) was added and the contents of the tube were allowed to stand for $\frac{1}{2}$ hr., during which time they were stirred three times with a glass rod. The reaction mixture was then transferred to a conical centrifuge tube and the carbonized residue separated by centrifuging for 20 min. at 3000 rev./min., swing-out buckets being used.

Duplicate 3 ml. samples of the clear supernatant were pipetted into thin-walled 16 mm. × 150 mm. Pyrex test tubes. To each of these samples were added 1 ml. of water, 0·5 ml. of arsenious acid solution and 1 ml. of 3·5 N-H_2SO_4, in that order, and the contents of the tubes mixed by 'flicking' as described below. The tubes were then incubated for 10 min. at 37 ± 0·1° in a water bath. When this period had elapsed, 0·5 ml. of ceric ammonium sulphate solution at 37° was added and the contents of the tube were mixed quickly. The most satisfactory way of carrying out the latter operation was found to be the following: the tube is held just below its rim in the left hand and the bottom of the tube flicked with the four fingers of the right hand in quick succession, the movement being repeated about 15 times.

Exactly 12·5 min. after the time when the addition of the ceric ammonium sulphate was begun, the extinction at 415 mμ of the solution contained in a 1 cm. cuvette was measured in a prism spectrophotometer (SP. 600, Unicam Instruments Ltd., Cambridge). Filling of the cuvette was begun 45 sec. before this time. It was found convenient to allow 1 min. between successive additions of ceric ammonium sulphate. This enabled a maximum of six serum samples to undergo iodide estimation in one batch, since duplicate iodide determinations were carried out on each sample.

The amount of 'added iodide' (i.e. iodide present in excess of that contaminating the reagents) in the reaction mixture was read from a calibration curve, connecting $E_{1\,\mathrm{cm.}}$ at 415 mμ with μg. of 'added iodide', whose derivation is described below. Multiplication of the figure thus obtained by 700/3 will give the result in μg. of I/100 ml. of serum.

Reagent blanks. A reagent blank was run with each group of estimations, 1 ml. of water replacing the 1 ml. sample of serum, and the entire analytical procedure being carried out as with serum. The reagents have been found sometimes to take up small amounts of iodine on keeping.

Construction of the calibration curve. The calibration curve was constructed by adding known amounts of iodide to reagent blanks. This was most easily done during preparation of the final solutions for iodide estimation. In place of 1 ml. of water, duplicate 1 ml. samples of solutions containing respectively 0·02, 0·04, 0·06, 0·08 and 0·10 μg. of I′/ml. were added to tubes containing 3 ml. of the eluate from a reagent

blank; a reagent blank containing no added iodide provided the zero point. The reduction of the ceric ion was then initiated and measurements were carried out as described above for the serum samples. The mean value of $E_{1\,cm.}$ was plotted on semi-logarithmic graph paper against μg. of 'added iodide' and a straight-line curve drawn to express the relation between them (see Fig. 2*c*). A fresh curve was constructed whenever a fresh solution of any reagent was made up.

Variants in method of PBI determination

Precipitation of PBI with trichloroacetic acid. Trichloroacetic acid precipitation was carried out according to Tong, Taurog & Chaikoff (1954). A serum sample (1 ml.) was added to 1 ml. of 20% (w/v) trichloroacetic acid (TCA), followed by 3 ml. of 10% (w/v) TCA and the suspension was thoroughly stirred with a glass rod. The precipitate was centrifuged off, washed three times in the centrifuge with 10 ml. of 2·5% (w/v) TCA and dissolved with stirring in 1 ml. of 4 N-Na_2CO_3, the glass rod being washed down with 1 ml. of water. The subsequent analytical procedure was the same as for $Zn(OH)_2$ precipitation.

Calibration curves and reagent blanks for solutions containing no zinc. Solutions for constructing calibration curves appropriate to conditions in which Zn^{2+} and TCA are absent were prepared from reagent blanks, consisting of 1 ml. of 4 N-Na_2CO_3 and 1 ml. of water, treated in the same way as has been described for $Zn(OH)_2$ precipitates. With TCA precipitation the reagent blanks consisted of 1 ml. of 4 N-Na_2CO_3, 0·5 ml. of 2·5% (w/v) TCA and 1 ml. of water, and were dried and ashed with the precipitated protein. Reagent blanks of this composition were used to prepare the iodide standards.

Elution of iodide after ashing of protein. (1) *Method according to Barker* et al. (1951). To the ash were added successively 2 ml. of 2 N-HCl, 2 ml. of 7 N-H_2SO_4 and 3 ml. of water, the suspension being stirred vigorously after each addition and the residue was centrifuged off when evolution of H_2S had ceased. Subsequent steps were the same as in the definitive method described above.

(2) *Method according to Brown* et al. (1953). The reagent volumes were modified in order to make the final dilution of eluate correspond with that used by Barker *et al.* (1951). To the ash was added 2·5 ml. of 2 N-HCl followed by 4·5 ml. of water. The residue was centrifuged off when evolution of H_2S had ended and the remaining steps of the analysis were as described above.

In both these modifications, reagent blanks, and consequently the solutions used for constructing calibration curves, were treated in the same manner as the ashed serum.

Chromatography of plasma extracts

Extracts were made from a total of five samples of plasma (containing 0·1 mg. of heparin/ml.) from five different patients suffering from thyrotoxicosis. The samples were obtained 48–96 hr. after the administration by mouth of therapeutic doses of radio-iodine (4–20 mc.). Plasma proteins were precipitated with $ZnSO_4$ and NaOH, as described above, except that the protein suspension was shaken vigorously by hand in a stoppered container, and the washed precipitate was suspended in 5 ml. of water. The suspension was acidified to pH 4 with 2 N-HCl, thereby redissolving the protein, and extracted three times with 10 ml. portions of water-saturated *n*-butanol. The *n*-butanol extracts were combined and evaporated to dryness under reduced pressure at 80° and the residue was taken up in 0·5 ml. of *n*-butanol. *n*-Butanol extracts were prepared in the same way from the combined supernatant and washings.

Duplicate samples (50 μl.) of the extracts were placed, 10 μl. at a time, on Whatman no. 1 paper, and chromatographed by the descending method in *n*-butanol–dioxan–2 N-NH_3 soln. (4:1:5, by vol.) as described by Gross, Leblond, Franklin & Quastel (1950). Carrier quantities (25 μg.) of non-radioactive thyroxine, di-iodotyrosine and tri-iodothyronine were added to the spots before developing the chromatogram. The amino acids were detected by spraying the paper with 0·2% ninhydrin in water-saturated *n*-butanol and heating it at 90° for 5 min.

The chromatogram was cut into strips 2 cm. wide, each strip corresponding to a different run, and the radioactivity present in each 1 cm. length was measured with a previously described apparatus (Acland, 1952). Each section of paper was counted for 5 min.

Recovery of PBI from serum

DL-Thyroxine solution was prepared by dissolving 157·3 mg. of the sodium salt in water, to which 2 ml. of N-NaOH had been added to increase the solubility of the amino acid, and diluting to 500 ml. with water. This solution was then diluted 1:200 with water. Both solutions were freshly made up when required. To 1 ml. of serum was added 0·1 ml. of the dilute thyroxine solution and the mixture incubated at 37° for 2 hr. The normal analytical procedure was then followed. The amount of thyroxine thus added to each serum sample increased its PBI concentration by 10 μg. of I/100 ml.

RESULTS

Iodide estimation by the reaction $Ce^{4+} \rightarrow Ce^{3+}$

The use of the reaction between ceric salts and arsenious acid for iodide estimation (Sandell & Kolthoff, 1937) depends upon the fact that the reduction of the ceric ion is normally a pseudo-unimolecular reaction, the rate-limiting process being the reaction between ceric ions and iodide (Glasstone, 1948). Hence, at constant temperature, the kinetic equation for a reaction of the first order can be applied. The catalysis of this reaction by iodide is an example of homogeneous catalysis in solution. Hence we can write (Glasstone, 1948):

$$k = k' + k_1 C_1, \qquad (1)$$

where k is the specific reaction rate for the reduction of ceric ion, k' is a constant, giving the specific reaction rate in the absence of 'added iodide' (added iodide is defined as iodide in excess of that contaminating the reagents), k_1 is the catalytic coefficient for 'added iodide' and C_1 is the concentration of 'added iodide'. Therefore

$$k' + k_1 C_1 = \frac{2{\cdot}303}{t} \log_{10}\left(\frac{a}{a-x}\right), \qquad (2)$$

where a is the initial concentration of ceric ion, and $(a-x)$ the concentration after time t. It follows

that, if t and a are constant, there is a linear relation between C_I and $\log_{10}(a-x)$. These conditions are fulfilled in the analytical procedure described above. The extinction at 415 mμ can be substituted for the ceric ion concentration, provided that the two are linearly related. Hence a linear relation between extinction and iodide concentration should result if the graph is plotted on semi-logarithmic paper. A kinetic approach, somewhat similar to the above, has been used by Caraway (1952) to eliminate the use of graphs in the method of Barker *et al.* (1951).

Experiments were performed, as described below, to test the validity of the arguments put forward in this section.

Relationship between extinction at 415 mμ and ceric ion concentration. The optical density of ceric salts in solution has been widely assumed, usually tacitly but sometimes explicitly (by Caraway, 1952), to follow Beer's law. That this is not necessarily the case is shown by the curve derived by Chaney (1940), which differs significantly from a straight line. The relationship between extinction and ceric ion concentration was re-investigated by making a series of dilutions of the ceric ammonium sulphate solution prepared for the iodide estimation at a constant concentration of sulphuric acid. The extinction of each solution at 415 mμ was measured and Fig. 1 shows that Beer's law is strictly obeyed.

Effect of interfering substances on the kinetics of the reaction $Ce^{4+} \rightarrow Ce^{3+}$. Barker (1948) has shown that chloride, while not itself catalysing the reduction of ceric salts by arsenious acid, increases the catalytic effect of iodide. Klein (1954) has demonstrated that sulphate decreases the catalytic effect of iodide. Experiments were performed to determine how, if at all, the kinetics of the reaction, $Ce^{4+} \rightarrow Ce^{3+}$, were affected by the presence of these ions, and also of zinc, whose influence on the reaction had not previously been studied.

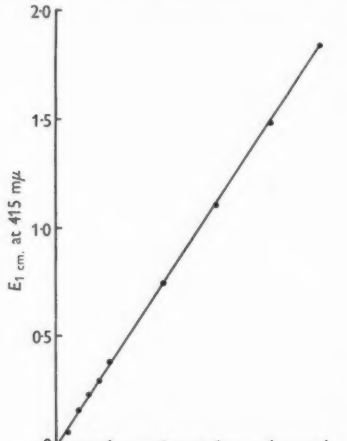


Fig. 1. Relation between extinction at 415 mμ and concentration of ceric ammonium sulphate in 0·29 N-H_2SO_4.

Calibration curves were constructed relating iodide concentration to extinction at 415 mμ, both in the presence and in the absence of zinc. The solutions used for constructing these curves were prepared by adding known amounts of iodide to reagent blanks, as described in the Experimental section. The procedure was repeated with the reagents appropriate to each of the three methods of dissolving iodide from the ashed-protein precipitate which were tested, namely those of Barker *et al.* (1951), Brown *et al.* (1953) and Grossmann & Grossmann (1955), the last-named being described as part of the definitive method in the Experimental section. Fig. 2 shows that the expected linear relation between $\log_{10} E$ and iodide concentration is obtained in each case if all the reagents used in the analytical procedure, other than zinc, are present. In the presence of zinc, however, in the first two methods there is a decrease in the catalytic effect of iodide and the curve shows a departure from linearity (see Fig. 2*a*, *b*). It should be noted that in both the above cases, iodide is eluted from the ash in acid conditions, hence all the zinc passes into solution. Grossmann & Grossmann (1955) use an eluent which remains alkaline, and zinc is left behind in the residue. With this technique the addition of either zinc or trichloroacetic acid to the reagent blanks does not affect the calibration curve for the determination of iodide (see Fig. 2*c*). At the four lower iodide concentrations, the extinction values obtained for the three different solutions were too close together to be plotted separately.

The non-linearity of the calibration curve in the presence of zinc, with acid eluents, was further studied in the following experiment. Two series of iodide standards containing 0·1 μg. of added iodide were prepared from reagent blanks by the elution method of Barker *et al.* (1951). One series of tubes contained zinc and the other did not, and the kinetics of the reaction, $Ce^{4+} \rightarrow Ce^{3+}$, in the two conditions were investigated by plotting $E_{1\,cm.}$ at 415 mμ against reaction time on semi-logarithmic graph paper (see Fig. 3). Each point represents the average of duplicate determinations in different tubes. First-order reaction kinetics are followed both in the presence and in the absence of zinc for approximately the first 15 min. Hence the non-linearity of the calibration curves obtained in the presence of zinc after a reaction time of 12·5 min. (Fig. 2*a*, *b*) would appear to have arisen because the decrease in the catalytic coefficient of iodide caused by this ion is greater at higher iodide concentrations.

Extraction of iodide from ashed serum protein

Recent workers have chosen to acidify immediately the highly alkaline ash that remains after heating dried serum protein with sodium carbonate, in order to break up the residue and to make its soluble components easy to dissolve out (Barker *et al.* 1951; Brown *et al.* 1953). However, Grossmann & Grossmann (1955), although treating the residue with acid in order to break it up, do not add sufficient to neutralize all the alkali present. Acidification is delayed until the final stages of the iodide estimation. The Experimental section contains details of the above methods, which were modified slightly for the purposes of the present investigation, and the results of comparative tests undertaken to determine which was the best of the three procedures for dissolving iodide from the ash are given in Table 1. All determinations were carried out on samples from the same specimen of normal serum in a $2\frac{1}{2}$ hr. ashing period. The highest estimate of the mean PBI concentration was observed when the extraction method of Grossmann & Grossmann (1955) was used. After extraction according to Barker *et al.* (1951) and Brown *et al.* (1953) some results were found to be below what has been stated to be the range in normal men: 4–8 μg. of PBI/100 ml. of serum (Roche & Michel, 1955). Furthermore, a coefficient of variation of less than 5 % was observed only when the elution method of Grossmann & Grossmann (1955) was employed. The last-named technique was consequently adopted when other stages of the analysis were tested. It is described in the Experimental section as part of the recommended procedure, whose overall efficiency was investigated in experiments to be described later.

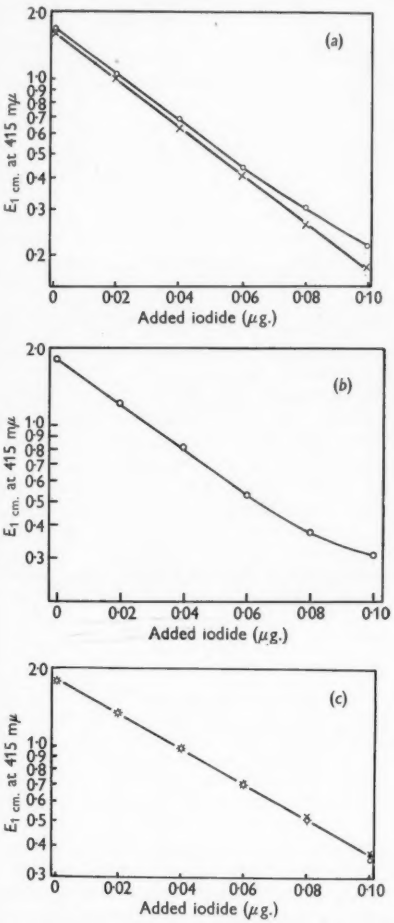


Fig. 2. Calibration of the reaction between ceric and arsenite ions for iodide determination. Relationship between $E_{1\,cm.}$ at 415 mμ after 12·5 min. reaction at 37° and weight of iodide added to the eluates from ashed reagent blanks containing protein precipitants. (*a*) Elution method of Barker *et al.* (1951); (*b*) method of Brown *et al.* (1953); (*c*) method of Grossmann & Grossmann (1955). Reagent blanks: ×, 2N-Na_2CO_3 (2 ml.); ○, 2N-Na_2CO_3 (2 ml.) and $Zn(OH)_2$ [≡1 ml. of 10% (w/v) $ZnSO_4,7H_2O$]; +, 2N-Na_2CO_3 (2 ml.) and 0·5 ml. of 2·5% (w/v) trichloroacetic acid.

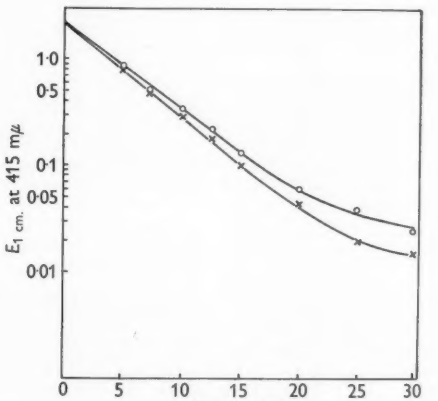


Fig. 3. Kinetics of the reaction between ceric and arsenite ions in the presence of 0·1 μg. of iodide added to the eluate from ashed reagent blanks, the elution method of Barker *et al.* (1951) being used. Symbols for reagent blanks are as in Fig. 2.

Table 1. *Estimated PBI concentration in the same serum specimen (1 ml. samples) with different methods being used to dissolve iodide from ashed protein*

Method	Eluent (ml.) 2N-HCl	7N-H_2SO_4	Water	No. of determinations	PBI concn. (μg. of I/100 ml.) Mean	Range
Barker *et al.* (1951)	2·0	2·0	3·0	62	2·9±1·08 (S.D.)	0·63–4·67
Brown *et al.* (1953)	2·5	—	4·5	11	4·5±0·88 (S.D.)	2·03–5·18
Grossmann & Grossmann (1955)	1·0	—	6·0	11	5·2±0·215 (S.D.)	4·78–5·60

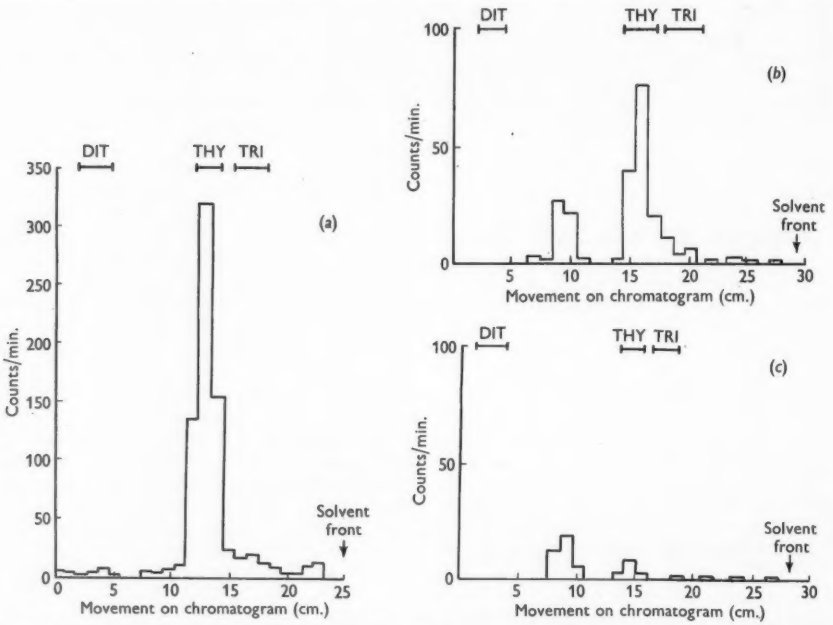


Fig. 4. Efficiency of PBI precipitation demonstrated by chromatograms prepared from the plasma of a thyrotoxic patient treated with ^{131}I. The distribution of radioactivity and the position of amino acids added as carriers are shown. (*a*) Protein precipitate from 1 ml. of plasma precipitated with 1 ml. of 10% (w/v) $ZnSO_4,7H_2O$ and 1 ml. of 0·5N-NaOH. (*b*) Supernatant and washings from (*a*). (*c*) Supernatant and washings from 1 ml. of plasma precipitated with 0·5 ml. of 10% (w/v) $ZnSO_4,7H_2O$ and 0·5 ml. of 0·5N-NaOH. DIT, 3:5-di-iodotyrosine; THY, thyroxine; TRI, 3:5:3′-tri-iodothyronine. The peak of radioactivity in (*b*) and (*c*), with R_F approx. 0·3, is attributed to inorganic iodide.

Precipitation and ashing of PBI

The efficiency of the precipitation of PBI with zinc hydroxide was studied both chromatographically and by chemical methods. Zinc hydroxide and trichloroacetic acid precipitation were compared by means of repeated analyses on samples from the same serum specimen.

Thyroxine, in combination with serum or plasma protein, was thought to provide a suitable standard for testing the efficiency of the method since it is the largest component of serum PBI. Reproducibility formed a further criterion for the chemical determinations. It was considered necessary that the coefficient of variation of the method should be less than 5% and that there should be no demonstrable difference between batches of analyses carried out on the same specimen at different times. No direct estimates of accuracy could be made since the serum specimens were not analysed by an independent method of known performance.

Efficiency of PBI precipitation. Chromatographic analysis of extracts, prepared as described above from the plasma of thyrotoxic patients who had been treated with radio-iodine, showed the following results. In the five samples studied, 2·5, 3, 3·5, 2 and

Table 2. *Results of seventy-five determinations of the PBI content of a specimen of normal serum*

PBI content is expressed as μg. of I/100 ml. Ashing time was 2½ hr.

Batch ...	1	2	3	4	5	6	7	8	9
No. of determinations	10	11	9	5	10	7	11	8	4
Mean	6·28	5·76	5·39	5·81	6·01	5·71	5·69	5·75	6·02
Range	6·00–6·42	5·51–6·18	5·13–5·67	5·67–6·13	5·70–6·16	5·25–6·23	5·48–5·83	5·53–5·99	5·90–6·23

1 % respectively of the total radioactivity associated with thyroxine was present in the combined supernatant and washings. The results obtained from the first serum are shown graphically in Fig. 4. Despite very vigorous shaking no difficulty was experienced in centrifuging down the precipitate, contrary to the findings of Barker *et al.* (1951). However, a further portion of the first serum was precipitated with half the usual concentration of zinc hydroxide. The washed precipitate was difficult to centrifuge down and Fig. 4*c* shows that 22 % of the thyroxine remained in the supernatant. In the protein precipitate, a small amount of radioactivity was associated chromatographically with tri-iodothyronine (about 5 % of that present in the thyroxine area). In no case did the combined supernatant and washings show radioactivity in the tri-iodothyronine area, the thyroxine spot alone among the amino acids containing radioactivity. The additional peak in Figs. 4*b* and 4*c*, with R_F approx. 0·3, is in the position where carrier-free radio-iodide was found in the same chromatographic system (Acland, 1952). This peak can therefore reasonably be identified with inorganic iodide, although further investigation with the ascending method and the same solvents gave an R_F for radio-iodide, both with and without carrier (50 μg. of KI), of 0·4–0·45. The R_F values in Fig. 4*b* are smaller than those in Fig. 4*c*, perhaps because of the higher salt concentration in the former extract.

In view of the errors inherent in the method of measuring the radioactivity in the chromatograms, the results quoted above cannot be more than semi-quantitative. It can, however, be concluded from them that large losses of endogenous PBI are unlikely to occur during protein precipitation with zinc hydroxide, provided that the method described in the Experimental section is adhered to.

Efficiency of the ashing procedure. A series of seventy-five determinations was carried out on a serum specimen obtained from a euthyroid subject (see Table 2). From these results a mean of 5·8 μg. of PBI/100 ml. and a standard deviation of ±0·31 μg. of PBI/100 ml. were calculated. In a further series of eight experiments, 0·1 μg. of iodine was added as the sodium salt of DL-thyroxine to 1 ml. samples of the same serum. The mean recovery was 96·5 % with standard deviation of ±2·1 % (range 93·5–99·8 %). Superficially, these results, which were quoted in a preliminary communication (Acland, 1956), seem satisfactory. However, on inspection of Table 2, the difference between batches of results which were obtained on different days appears larger than one would expect from the scatter of values within the same batch. This impression was strengthened by an analysis of variance which showed that the 'between-batch' variance was significantly greater than the 'within-batch' variance ($P \ll 0{\cdot}001$).

Table 3. *Comparison between zinc hydroxide and trichloroacetic acid as precipitants of PBI in samples from the same serum specimen*

Serum PBI concn. is in μg. of I/100 ml. Each result is the mean of five determinations.

Ashing time (hr.)	$Zn(OH)_2$ precipitation		Trichloroacetic acid precipitation	
	Mean	Range	Mean	Range
2½	5·64	5·39–5·79	5·43	5·25–5·76
	4·94	4·78–5·02	5·47	4·78–5·95
	5·32	5·13–5·53	5·03	3·97–5·48
	4·84	4·78–4·90	5·46	5·06–5·67
	4·85	4·67–5·13	5·20	4·78–5·53
3½	5·10	4·83–5·44	5·15	4·78–5·53
	5·03	4·90–5·24	5·19	5·13–5·20
	4·79	4·60–5·02	5·11	4·85–5·44

As described below, this difference between batches was finally attributed to a failure in thermostatic control. Before this conclusion was reached, however, some experiments were carried out to determine whether trichloroacetic acid was a more satisfactory reagent for precipitating PBI than zinc hydroxide. These will be described next.

Batches of ten samples were taken from a serum specimen, five of which were precipitated by zinc hydroxide and five by trichloroacetic acid. The washed precipitates were ashed for 2½ hr. The results of five such batches of determinations are given in Table 3.

With zinc hydroxide precipitation, the mean serum PBI concentration was found to be 5·1 μg./100 ml. and the standard deviation ±0·35 μg./100 ml. For trichloroacetic acid precipitates, corresponding figures were 5·3 ± 0·41 μg./100 ml. Separate analyses of variance were carried out on the results of each precipitation method.

The difference between the means of batches precipitated by zinc hydroxide was again found to be statistically significant in an analysis of variance ($P \ll 0{\cdot}001$). This 'between-batch' difference was not found when trichloroacetic acid was used as a precipitant. The latter method, however, shows greater variability within batches than the former. This difference in variability was found to be statistically significant ($P < 0{\cdot}002$ in a two-tailed F test).

It was noticed that some of the finely divided, carbonized residue, derived from trichloroacetic acid precipitates, was not sedimenting on centrifuging and it was thought that this possible source of error might be eliminated by varying the ashing conditions. Three further batches of ten analyses were carried out with a 3½ hr. ashing time on the same serum sample which had been ashed for 2½ hr. Once again, in each batch five determinations were made on zinc hydroxide precipitates and five on trichloroacetic acid precipitates. The carbonized residue from the trichloroacetic acid precipitates centrifuged down completely. The results of this experiment are given in Table 3. The difference between batches of determinations on zinc hydroxide precipitates is again statistically significant ($0{\cdot}01 < P < 0{\cdot}05$). The 'within-batch' variance shown by trichloroacetic acid precipitates is decreased by increasing the ashing time. Comparison of the 'within-batch' variances for trichloroacetic acid precipitates calculated from the data used to construct Table 3 showed that this decrease was statistically significant ($0{\cdot}02 < P < 0{\cdot}05$ in a two-tailed F test).

At this stage in the investigation it was noticed that the thermostat controlling the furnace used for ashing was behaving erratically, sometimes allowing the furnace temperature to rise above 650°. An experiment was then carried out to examine the effect of varying the ashing temperature on the analytical results. A new serum specimen was obtained and PBI determinations were carried out on five batches of samples, each batch containing some zinc hydroxide precipitates and some trichloroacetic acid precipitates. The zinc hydroxide precipitates were allowed to stand for 3 hr. before centrifuging, as recommended by Skanse & Hedenskog (1955), although no experiments were carried out to confirm the value of this procedure. For these determinations, the ashing temperature was not allowed to rise above 610°. A further two batches were ashed at 650°. An ashing time of 3½ hr. was used, although the superiority of the longer ashing time was not demonstrated with zinc hydroxide precipitates. With trichloroacetic acid precipitates, ashed at the lower temperature, some of the finely divided residue did not sediment in the centrifuge. The results of this experiment are given in Table 4. Analysis of variance of the data for the samples ashed at 590–610° showed that the difference between batches of determinations on zinc hydroxide precipitates was no longer statistically significant. All the results can therefore legitimately be combined to give a grand mean of 4·3 μg. of PBI/100 ml. and a standard deviation of $\pm 0{\cdot}17$ μg. of PBI/100 ml. The two batches of determinations on zinc hydroxide precipitates ashed at 640–660° give a grand mean of 4 μg. of PBI/100 ml. The difference between these two means is statistically significant ($t = 2{\cdot}96$; $0{\cdot}001 < P < 0{\cdot}01$). It seems likely, therefore, that inadequate thermostasis during ashing was responsible for the previously observed differences between batches of analyses on zinc hydroxide precipitates and for the higher value previously reported (Acland, 1956) for the variability of the method.

The results of determinations on trichloroacetic acid precipitates are erratic. In the series of analyses with the lower ashing temperature, there is a statistically significant difference between batches. On inspection of Table 4, the values are seen to decrease steadily from batch to batch. In addition, some of the determinations on precipitates ashed at the higher temperature show considerable losses.

It can be concluded that apparent losses of PBI from trichloroacetic acid precipitates are likely to occur if the ashing temperature rises above 650°. At lower temperatures, ashing for 3½ hr. is inadequate

Table 4. *Effect of raised ashing temperature on the estimated PBI content of a serum specimen for both zinc hydroxide and trichloroacetic acid precipitation*

Results are in μg. of PBI/100 ml.

		Ashing temperature 590–610°					Ashing temperature 640–660°	
$Zn(OH)_2$ precipitation	No. of determinations	5	5	3	5	5	5	4
	Mean	4·34	4·31	4·42	4·12	4·20	4·12	3·93
	Range	4·27–4·50	4·27–4·36	4·36–4·48	3·97–4·25	4·04–4·67	3·99–4·32	3·64–4·11
Trichloroacetic acid precipitation	No. of determinations	5	5	5	4	5	5	5
	Mean	4·80	4·60	4·35	4·02	3·98	4·15	3·42
	Range	4·62–5·04	4·43–4·71	4·08–4·54	3·87–4·32	3·70–4·48	3·81–4·43	3·30–3·66

to oxidize all the finely divided, carbonized particles in the residue. Although a longer period of ashing might possibly give better results it would, in these circumstances, be inconvenient to carry out ashing and iodide determination on the same day. It was felt that since zinc hydroxide precipitation gave adequate results, it was not worth while to investigate further the feasibility of using trichloroacetic acid as a precipitant.

Recovery experiments

A series of five determinations was made with zinc hydroxide precipitation. Five samples were taken from the same serum specimen as was used to obtain the data in Table 4. To each 1 ml. sample was added 0·1 μg. of iodine as DL-thyroxine. The percentage of iodine recovered in these experiments was respectively 89·1, 96·6, 94·7, 92·6 and 91·5, giving a mean recovery of 92·9 %.

In a further series of five experiments, the percentage recovery of thyroxine, equivalent to 0·1 μg. of iodine, added to the washed zinc hydroxide precipitates from reagent blanks, was respectively 75·8, 80·3, 77·0, 75·8 and 74·7, giving a mean recovery of 76·7 %. Combination of organic iodine with protein would thus appear to prevent excessive losses of iodine in the ashing procedure.

DISCUSSION

Estimation of iodide by the reaction $Ce^{4+} \rightarrow Ce^{3+}$

The theoretical basis for using the reaction between ceric and arsenite ions to estimate iodide has been described above. It was shown that there are three prerequisites for the reaction to be applied in optimum conditions. First, ceric ammonium sulphate solutions must obey Beer's law. Secondly, I' the reaction $Ce^{4+} \rightarrow Ce^{3+}$ must be rate-limiting. Thirdly, the catalytic coefficient for iodide must be constant over the whole useful range of iodide concentrations.

Some comment is necessary on the relation between extinction and ceric ion concentration. Chaney (1940) obtained a curve very far from a straight line for this relationship. However, he used a Corning no. 554 filter in conjunction with a photometer adapted for test tubes. A linear relation is more likely to be observed if, as in the present investigation, monochromatic light and cuvettes with optically flat surfaces are used (Kirk, 1950). Thompson, Klugerman & Truemper (1956) found that only the Beckmann DU spectrophotometer, of several tried, gave adequate results. Fig. 1 shows that the Unicam model SP. 600 has a comparable performance.

Some confusion appears to exist in the literature about the applicability of Beer's law to iodide estimation by the reaction $Ce^{4+} \rightarrow Ce^{3+}$. The law can, of course, refer only to the relation between extinction and ceric ion concentration, not to that between extinction and iodide concentration as suggested by Klein (1954) and by Grossmann & Grossmann (1955). The latter relation is shown above to be a logarithmic one. It follows that Grossmann & Grossmann's (1955) linear-calibration curve, relating difference in extinction to iodide concentration, has no theoretical basis.

Fig. 3 demonstrates that first-order kinetics hold for a reaction time of 12·5 min., which is that used in the analysis. Hence the reduction of ceric ion is rate-limiting. Similar curves are shown by Thompson *et al.* (1956).

Figs. 2 and 3 show that zinc ion lowers the catalytic coefficient for iodide, this decrease being proportionately greater at higher concentrations. The reduction of the ceric ion follows first-order kinetics in the presence of this ion for as long a reaction time as in its absence. It should be noted that the original procedure employed by Barker *et al.* (1951) does not compensate for interference of this type. The addition of a known amount of iodide to an unknown will cause, in such a case, a smaller proportionate increase in the rate of reaction as the iodide concentration in the unknown becomes greater, while the relationship between $\log_{10} E$ and this time remains rectilinear. The errors thus caused with zinc are not of practical importance since their effect is to underestimate, by not more than about 10 %, the PBI content of sera whose PBI concentration is greater than about 10 μg./100 ml., well above the normal range. In any case, Barker (personal communication) now recommends '...that a calibration curve be employed rather than the cumbersome addition of a standard amount of iodide to each unknown'. He further states: 'The main reason this latter procedure was originally instituted was our concern over possible interference from substances in plasma or serum acting like mercury to alter the catalytic effect of iodide. Although mercurial diuretics will occasionally give such an effect, usually a one-day wait is adequate for elimination of the mercury from the bloodstream.' However, Grossmann & Grossmann's (1955) elution method is preferable because it eliminates interference by zinc and results in a theoretical straight-line calibration curve (see Fig. 2*c*).

Elution of iodide from ashed protein

The highly acid solutions used by Barker *et al.* (1951) and by Brown *et al.* (1953) to dissolve iodide from ashed protein were shown (Table 1) to result in losses of iodide. It follows that the normal limiting values for serum PBI concentration given by these workers are likely to be too low. A re-determination

of these limits would consequently appear to be necessary. Acidification after separation of the supernatant from the residue, as used in the method of Grossmann & Grossmann (1955), was shown to be satisfactory and was finally adopted with slight modifications. Acidification by itself does not therefore cause a loss of iodide. It is probable that acid conditions during elution favour the conversion of iodide into iodine and its retention by the carbonized residue derived from serum protein or on the walls of the Pyrex tube.

Precipitation and recovery of protein-bound iodine

From the results of the serial chemical determinations described above it is reasonable to conclude that, in the long run, with the same analytical method the percentage of added thyroxine recovered should be in the region of 90–95 %. According to Tong *et al.* (1954) only 90 % of [^{131}I]thyroxine added to serum is recovered in a zinc hydroxide precipitation. The chromatographic experiments reported here appear to indicate losses of not more than about 5 % in this stage of the analysis. It may be that some of the radioactivity in the labelled thyroxine used by Tong *et al.* (1954) was present in the form of inorganic iodide.

The finding that apparent losses of iodine occur during the analysis when thyroxine is not associated with serum protein makes it unlikely that the method can be usefully applied to the determination of organic iodine in the absence of a large excess of protein. The protection afforded by protein may perhaps result from the adsorption of iodide on the carbonized residue, which might prevent occlusion in the walls of the combustion tubes. Losses of this type are perhaps more liable to occur at higher ashing temperatures, since sodium iodide melts at 651° and zinc iodide boils at 624°. It is possible that the bulkier zinc hydroxide precipitates are less liable than trichloroacetic acid precipitates to lose iodide into the walls of the combustion tubes between 590 and 610°. On the other hand, zinc hydroxide precipitates may lose iodide more rapidly than trichloroacetic acid precipitates between 625 and 650° as a result of the volatilization of zinc iodide. Above 650°, the melting of sodium iodide may increase the losses from trichloroacetic acid precipitates.

PBI estimations are not very specific chemically. There is probably no discrimination between the different iodothyronines. In addition, about 65 % of di-iodotyrosine added to plasma co-precipitates with protein when zinc hydroxide, but not trichloroacetic acid, is used as precipitant (Tong *et al.* 1954). No work has been reported on the co-precipitation with plasma proteins of mono-iodotyrosine, reported to be present together with di-iodotyrosine in the blood of a goitrous patient by Stanbury, Kassenaar, Meijer & Terpstra (1955). According to Taurog, Wheat & Chaikoff (1956) appreciable amounts of inorganic iodide (2–3 %) may remain in a washed trichloroacetic acid precipitate, and therapy with potassium iodide is known to give rise to spuriously high analytical results when zinc hydroxide precipitation of PBI is used (Barker *et al.* 1951). Iodine in certain abnormal-serum components would also appear as PBI. Such compounds are thyroglobulin, found in blood after radio-iodine therapy (Robbins, 1954), and a serum-albumin component described by Robbins, Rall & Rawson (1955) in a patient with thyroid carcinoma. It is not yet certain how accurately the iodine content of these proteins would be estimated by ordinary methods of PBI determination. Finally, intravenous injection of radio-opaque substances containing iodine for special X-ray examinations causes the serum PBI to remain raised for a period of years (Barker *et al.* 1951).

Comparison of the present method with other methods of determining PBI

Methods of determining PBI based on the alkaline-ashing technique for converting organically bound iodine into iodide are preferable for routine use to those using acid digestion and distillation, since the former type requires less supervision and less stringent technical precautions (Barker & Humphrey, 1950). In addition, two well-known examples of distillation methods, those of Barker (1948) and Taurog & Chaikoff (1946), involve losses of iodine of the order of 10–15 % during distillation.

Table 5 shows the reports of some other workers on the reliability of their methods. Results comparable to those reported here are claimed by Sobel & Sapsin (1952), using both their own and Chaney's (1940) acid-distillation methods, by Skanse & Hedenskog (1955), using a modification of the Barker *et al.* (1951) method, and by Thompson *et al.* (1956), using another modification of the same method. However, Chaney's (1940) method requires 3 ml. of serum and the distillation is time-consuming, whereas the results of Sobel & Sapsin's (1952) own method are stated by them to be affected if the door of the laboratory in which the estimations are being carried out is opened while the distillation process is proceeding. Thompson *et al.* (1956) have studied their own method with a quality-control technique, and find that it sometimes goes 'out of control'. These authors use the highly acid conditions which have been criticized above. Skanse & Hedenskog (1955) also use an acid solution to elute iodide from ashed protein, which probably accounts for their observing a 15 % loss of iodine during the ashing process. They allow for this loss in their calibration of the reaction but,

Table 5. *Reliability of some published methods of determining serum PBI*

Author(s)	Method	S.D. or coeff. of variation for a single estimation	Mean difference between duplicates (μg. of I/100 ml.)
Gottschalk & Riggs (1952)	Man, Smirnow, Gildea & Peters (1942)	±0·46 μg./100 ml.	—
Stanbury *et al.* (1954)	Barker (1948) modified	±0·39 μg./100 ml.	0·42
Sobel & Sapsin (1952)	Chaney (1940)	±4·0 %*	0·15
	Sobel & Sapsin (1952)	±7·7 %*	0·34
Caraway (1952)	Barker *et al.* (1951)	—	0·5
Skanse & Hedenskog (1955)	Barker *et al.* (1951) modified	±0·22 μg./100 ml.	—
Thompson *et al.* (1956)	Barker *et al.* (1951) modified	±5·7 %†	—
Acland (present work)	Barker *et al.* (1951) modified	0·17 μg./100 ml.	—

* Calculated from the 95 % range for the mean of duplicate estimations as given by the authors (±5·6 and ±10·9 % respectively).

† Calculated from the coefficient of variation for the mean of triplicate determinations as given by the authors (±3·3 %).

in view of the results with acid eluents which were obtained during the present investigation, it seems doubtful whether the loss would remain constant in the long run.

The possibility of stopping the reduction of the ceric ion at a fixed time, as recommended by Grossmann & Grossmann (1955) and by Meyer, Dickenman, White & Zak (1955), is attractive. It does, however, involve the running of simultaneous standards to counteract the effect of fading colour with time. It is simpler to read the iodide content directly off a calibration curve. No difficulty has been experienced in timing the extinction readings to within 1–2 sec. and the use of the same pipette for adding ceric ammonium sulphate has ensured constancy of delivery time.

The method of determining serum PBI described in the present communication seems sufficiently accurate and reliable to provide trustworthy results in a routine laboratory.

SUMMARY

1. A method of determining serum-protein-bound iodine (PBI) is described, which is suitable for routine use.

2. A series of twenty-three determinations on a serum specimen, with zinc hydroxide as a precipitant of protein-bound iodine, gave a mean of 4·3 μg. of PBI/100 ml. and a standard deviation of ±0·17 μg. of PBI/100 ml. The mean recovery of thyroxine (≡10 μg. of PBI/100 ml.) added to this serum specimen was 92·9 %.

3. Analyses on trichloroacetic acid precipitates did not give consistent results owing to difficulties experienced in the ashing process.

4. Losses of iodine result if the ashing temperature rises above 650°.

5. Acidifying a suspension of ashed serum protein is shown to cause losses of iodine. Elution of iodide from the ash is satisfactory in alkaline conditions.

6. The mean recovery of thyroxine added to reagent blanks before ashing was found to be only 76·7 %.

7. A linear calibration curve can be constructed for the reaction between ceric salts and arsenious acid, which relates the concentration of added iodide to the logarithm of the extinction measured at 415 mμ after the reaction has proceeded for 12·5 min.

8. Zinc is shown partly to inhibit the catalytic action of iodide on the reaction, $Ce^{4+} \rightarrow Ce^{3+}$, the effect being greater at higher iodide concentrations.

The author thanks Dr Arthur Jordan, Professors E. J. Wayne and G. M. Wilson for their interest in this work, Professor Q. H. Gibson for his helpful comments, Dr E. K. Blackburn and the staff of the Sheffield Regional Blood Transfusion Service for gifts of serum, Dr G. H. Jowett for advice on statistics and the Board of Governors of the United Sheffield Hospitals for financial assistance.

REFERENCES

Acland, J. D. (1952). *Nature, Lond.*, **170**, 32.
Acland, J. D. (1955). *J. Physiol.* **128**, 61 *P*.
Acland, J. D. (1956). *Biochem. J.* **62**, 30 P.
Barker, S. B. (1948). *J. biol. Chem.* **173**, 715.
Barker, S. B. & Humphrey, M. J. (1950). *J. clin. Endocrin.* **10**, 144.
Barker, S. B., Humphrey, M. J. & Soley, M. H. (1951). *J. clin. Invest.* **30**, 55.
Brown, H., Reingold, A. M. & Samson, M. (1953). *J. clin. Endocrin.* **13**, 444.
Caraway, W. T. (1952). *J. clin. Endocrin.* **12**, 1215.
Chaney, A. L. (1940). *Industr. Engng Chem. (Anal.)*, **12**, 179.
Deiss, W. P., Albright, E. C. & Larson, F. C. (1953). *Proc. Soc. exp. Biol., N.Y.*, **84**, 513.
Glasstone, S. (1948). *Textbook of Physical Chemistry*, 2nd (British) ed., pp. 1087, 1141. London: Macmillan.
Gordon, A. H., Gross, J., O'Connor, D. & Pitt-Rivers, R. (1952). *Nature, Lond.*, **169**, 19.
Gottschalk, C. W. & Riggs, D. S. (1952). *J. clin. Endocrin.* **12**, 235.
Gross, J., Leblond, C. P., Franklin, A. E. & Quastel, J. H. (1950). *Science*, **111**, 605.

Grossmann, A. & Grossmann, G. F. (1955). *J. clin. Endocrin.* **15**, 354.
Kirk, P. L. (1950). *Quantitative Ultramicroanalysis*, p. 66. New York: Wiley.
Klein, E. (1954). *Biochem. Z.* **326**, 9.
Man, E. B., Smirnow, A. E., Gildea, E. F. & Peters, J. D. (1942). *J. clin. Invest.* **21**, 773.
Meyer, K. R., Dickenman, R. C., White, E. G. & Zak, B. (1955). *Amer. J. clin. Path.* **25**, 1160.
Petermann, M. L., Robbins, J. & Hamilton, M. G. (1954). *J. biol. Chem.* **208**, 369.
Robbins, J. (1954). *J. biol. Chem.* **208**, 377.
Robbins, J., Petermann, M. L. & Rall, J. E. (1955). *J. biol. Chem.* **212**, 403.
Robbins, J., Rall, J. E. & Rawson, R. W. (1955). *J. clin. Endocrin.* **15**, 1315.
Roche, J. & Michel, R. (1955). *Physiol. Rev.* **35**, 583.
Roche, J., Michel, R., Wolf, W. & Nunez, J. (1956). *Biochim. biophys. Acta*, **19**, 308.
Salter, W. T. & McKay, E. A. (1944). *Endocrinology*, **35**, 380.
Sandell, E. B. & Kolthoff, I. M. (1937). *Mikrochem. Acta*, **1**, 9.
Skanse, B. & Hedenskog, I. (1955). *Scand. J. clin. Lab. Invest.* **7**, 291.
Sobel, H. & Sapsin, S. (1952). *Analyt. Chem.* **24**, 1829.
Stanbury, J. B., Brownell, G. L., Riggs, D. S., Perinetti, H., Itoiz, S. & Del Castillo, E. B. (1954). *Endemic Goiter. The Adaptation of Man to Iodine Deficiency*, p. 23. Cambridge, Mass.: Harvard University Press.
Stanbury, J. B., Kassenaar, A. A. H., Meijer, J. W. A. & Terpstra, J. (1955). *J. clin. Endocrin.* **15**, 1216.
Taurog, A. & Chaikoff, I. L. (1946). *J. biol. Chem.* **163**, 313.
Taurog, A. & Chaikoff, I. L. (1948). *J. biol. Chem.* **176**, 639.
Taurog, A., Wheat, J. D. & Chaikoff, I. L. (1956). *Endocrinology*, **58**, 121.
Thompson, H. C., Klugerman, M. R. & Truemper, J. (1956). *J. Lab. clin. Med.* **47**, 149.
Tong, W., Taurog, A. & Chaikoff, I. L. (1954). *J. biol. Chem.* **207**, 59.
Trevorrow, V. (1939). *J. biol. Chem.* **127**, 737.

Biochemistry of the Wood-rotting Fungi

9. VOLATILE METABOLIC PRODUCTS OF *STEREUM SUBPILEATUM* BERK. & CURT.*

By J. H. BIRKINSHAW, P. CHAPLEN and W. P. K. FINDLAY
Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London, and The Forest Products Research Laboratory, Department of Scientific and Industrial Research

(*Received* 12 *November* 1956)

Our researches on the volatile metabolic products of some of the wood-rotting fungi led us to examine an organism which was isolated from the staves of a beer barrel constructed of Persian oak, *Quercus castaneaefolia* C. A. Mey. A batch of about eighty casks made of Persian oak, after being in use for 2–5 years, developed a curious scented odour which contaminated the beer so badly that the casks had to be destroyed.

Examination of staves from the defective casks showed small internal pockets of incipient white rot. Isolations from these areas yielded a vigorous golden-orange culture of a basidiomycete (now listed as Forest Products Research Laboratory no. 271A) which was found to be identical with a culture of *Stereum subpileatum* originally isolated in the U.S.A. by the late L. O. Overholts of Pennsylvania State College. Later, sporophores of the fungus developed on portions of staves kept in a damp chamber, and the structure of these agrees well with the description of *S. subpileatum* Berk. & Curt. This fungus has been reported by Long (1915) as a common cause of a 'honeycomb' white-pocket rot in thirteen species of oak in the southern part of the U.S.A. He noted that the fungus can continue to grow in felled timber. It is highly probable that infection in the oak used for the manufacture of beer barrels was present at least in an incipient stage in the logs when they were felled.

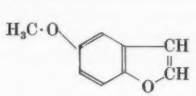
The production of a sweet musty odour by *S. subpileatum* has been described as a characteristic feature of this fungus. The odour from a culture is both penetrating and persistent and resembles a scent used in cheap soaps. It has been suggested that it may be compared with 'a mixture of wintergreen and apricots plus a trace of thymol'. There is little doubt that the metabolic products of the fungus growing in the wood were responsible for the contamination of the beer, even though the casks were lined inside with a vegetable pitch.

In order to investigate the metabolic products of *S. subpileatum* responsible for the characteristic odour, the fungus was cultured in the laboratory and the volatile products were separated from the mycelium and culture fluid by steam-distillation as in previous cases. As preliminary tests had shown that a carbonyl compound and an acid were present in the ether extract of the steam-distillate, the

* Part 8: Birkinshaw & Chaplen (1955).

distillate was extracted with ether and the ether solution was shaken with bisulphite and then with bicarbonate. The bisulphite solution, on acidification, afforded the free carbonyl compound, which was converted into the 2:4-dinitrophenylhydrazone. The carbonyl compound proved to be cinnamic aldehyde. Its identity was confirmed by preparation of the oxime. Acidification of the bicarbonate fraction afforded cinnamic acid, confirmed by mixed m.p. and by conversion into the dibromide.

The extracted ether solution contained a neutral crystalline product of m.p. 34°, having the characteristic odour of the cultures. It possessed the molecular formula $C_9H_8O_2$ and contained one methoxy group. It gave a negative reaction for carboxylic acid or ester and no colour with ferric chloride. The ultraviolet-absorption spectrum was close to that of coumarone; it was therefore probable that the substance was a methoxycoumarone. Of the known methoxycoumarones none has been reported as solid at room temperature except 5-methoxycoumarone (I). This substance was obtained in minute amount by Stoermer (1900) as an oil of b.p. 230–240°. He remarked on its peculiar sweet and clinging odour. It was later synthesized by Tanaka (1951), who records it as a solid of m.p. 32–33°.



(I)

The compound was synthesized by Tanaka's method and was found not to depress the m.p. of our natural product, on mixing. Confirmation of identity was sought by the action of bromine on the synthetic and natural products. It was expected that addition of bromine would occur at the double bond (2:3-addition) as with coumarone itself. Although the reaction was carried out at low temperature (−5°) in carbon disulphide with 2 atom-equivalents of bromine present, hydrobromic acid was evolved and two crystalline bromine compounds were obtained from both the synthetic and the natural product. These crystalline derivatives were (*a*) a colourless product, $C_9H_7O_2Br$, m.p. 76°, and (*b*) a yellow product, $C_9H_7O_2Br_3$, m.p. 80°. Evidently substitution had occurred in the benzene ring in both compounds and addition only in the yellow compound. The two products are therefore *x*-bromo-5-methoxycoumarone and 2:3:*x*-tribromo-5-methoxycoumaran respectively. The mono- and tri-bromo compounds from the natural 5-methoxycoumarone were identical with the mono- and tri-bromo compounds respectively obtained from the synthetic material (no depression in m.p. on mixing).

Cinnamic acid is known to occur in nature in various plant oils and resins. It is produced in the form of the methyl ester by one of the higher fungi, *Lentinus lepideus* (Birkinshaw & Findlay, 1940).

Cinnamaldehyde is a constituent of many essential oils (e.g. oil of cinnamon, cassia oil, patchouli oil) but has not hitherto been recorded as a fungal metabolic product. It may be the precursor of the cinnamic acid present in the cultures of *Stereum subpileatum*. The aldehyde could be converted into the acid by the action of a fungal enzyme or even by oxidation in the air.

5-Methoxycoumarone has not previously been reported as occurring in nature. Its production by a fungus recalls the hypothesis previously advanced that gentisic aldehyde is concerned in the fungal metabolic processes (Birkinshaw, 1953). It was suggested that it might be the common precursor of gentisyl alcohol and patulin, products of *Penicillium patulum*, as well as of gentisic acid, produced by *P. griseofulvum*. 5-Methoxy-2-hydroxybenzaldehyde derived by single *O*-methylation from gentisic aldehyde could, conceivably, by condensation with acetaldehyde and oxidation, be converted into 6-methoxycoumarin. The latter, by processes analogous to the chemical or thermal conversion of coumarin into coumarone, could then yield 5-methoxycoumarone. The oxygen methylation need not necessarily be the first step but could occur anywhere in the reaction chain. Cinnamic aldehyde (and hence cinnamic acid) could be derived by a similar condensation from benzaldehyde; this aldehyde, like gentisic aldehyde, has not yet been detected as a fungal product, but benzoic acid is known to be formed by *Penicillium roseo-purpureum* (Posternak, 1940).

EXPERIMENTAL

All m.p.'s are uncorrected. Elementary analyses are by Weiler and Strauss except where otherwise stated. Methoxy determinations were made by one of us (P.C.).

Organism

The culture used was F.P.R.L. no. 271A, of *Stereum subpileatum* Berk. & Curt., isolated from a Persian-oak beer-barrel stave.

Culture conditions

Two media were employed: (1) aqueous malt extract (Vimaltol; A. Wander Ltd., London, W. 1), 4% (pH 5·6). This was sterilized by autoclaving at 110° for 30 min. (2) A synthetic medium as used for *Daedalea juniperina* (Birkinshaw & Chaplen, 1955), containing only glucose, Marmite and inorganic salts. It was sterilized by steaming on 3 successive days.

The medium was distributed (350 ml./flask) in 1 l. conical flasks plugged with cotton wool. The flasks were sterilized and inoculated by placing on the surface of the liquid a fragment of mycelium derived from a malt-agar slope. The flasks were incubated in the dark at 24°.

Culture characteristics

On the malt medium the mycelium, after 6 weeks, was orange-yellow in colour with deep-brown reverse. It was of a leathery nature but somewhat scanty, and did not cover the whole surface of the liquid in the flask. The culture filtrate was deep brown in colour and turbid. On the synthetic medium growth was very sparse. The colour of the mycelium was creamy white, with very little orange tint. The culture solution was light greyish brown and turbid. In both cases the cultures soon developed the characteristic odour which was immediately perceptible on entering the incubation room. As can be seen from Table 1, the products were qualitatively the same on the two media.

Harvesting and separation of volatile products

The method employed for one batch of fifty flasks (malt medium) will be described; a similar method was used for the other two batches.

The total contents of the flasks were subjected to steam-distillation. The disillate was extracted four times with one-quarter of its volume of ether. The ether extracts were dried (Na_2SO_4) and the ether was removed by distillation. The residual brown syrup (3·1 g.) rapidly crystallized on cooling. It was dissolved in ether and extracted six times with an equal volume of freshly prepared saturated $NaHSO_3$ solution. The ether was washed with water and the washings were added to the bisulphite solution. The ether was shaken six times with an equal volume of freshly prepared saturated $NaHCO_3$ solution and washed with water. The extracted ether solution was then dried over Na_2SO_4.

Treatment of bisulphite extract; isolation of cinnamaldehyde

The bisulphite extract was strongly acidified with conc. HCl and extracted four times with one-quarter of its volume of ether. The ether extract was dried and the ether was removed. The residual brownish yellow oil (0·5 g.) deposited a few small crystals on chilling. A portion (0·1314 g.) of this residue, when dissolved in a little ethanol and treated with an excess of 2:4-dinitrophenylhydrazine hydrochloride in 2N-HCl, gave 0·2311 g. of an orange-red 2:4-dinitrophenylhydrazone of m.p. 247–249°. Crystallization from acetic acid, then ethyl acetate, afforded deep-red needles, m.p. 251–252° decomp. [Found: C, 57·6; H, 3·8; N, 17·5; OMe, nil. Calc. for $C_{15}H_{12}O_4N_4$ (i.e. the dinitrophenylhydrazone of C_9H_8O): C, 57·7; H, 3·9; N, 18·0%]. An authentic specimen of cinnamaldehyde 2:4-dinitrophenylhydrazone of m.p. 252° produced no depression in m.p. when mixed with the above derivative of the natural product.

Another portion (0·12 g.) of the oil recovered from the bisulphite extraction was dissolved in ethanol (3 ml.) and treated with a solution of hydroxylamine hydrochloride (0·15 g.) and anhydrous sodium acetate (0·3 g.) in water (5 ml.). After several hours, the brown ppt. (90 mg., m.p. 85–90°) was collected and recrystallized successively from light petroleum, aqueous ethanol and light petroleum. Long thick colourless needles (50 mg.) of m.p. 136° were obtained. These did not depress the m.p. of authentic cinnamaldehyde oxime, m.p. 137°. The presence of cinnamaldehyde in the volatile products was thus confirmed.

Treatment of the bicarbonate extract; isolation of cinnamic acid

The aqueous bicarbonate extract was acidified with HCl. Colourless leaflets (0·38 g.) separated, m.p. 132–133°. The filtrate was extracted with ether and further acid (80 mg., m.p. 128–130°) was thus recovered. Recrystallization from water afforded colourless leaflets (0·4 g., m.p. 132°) which did not depress the m.p. (133°) of an authentic specimen of cinnamic acid on admixture. [Found (Schoeller): C, 72·7; H, 5·4. Equivalent by titration, 146. Calc. for $C_9H_8O_2$: C, 72·9; H, 5·4%; equivalent, 148]. Further confirmation of the identity of this substance was obtained by converting it into the dibromide. The acid (25 mg.) was dissolved in CS_2 and treated with drops of bromine until the latter was no longer decolorized. After removal of CS_2 the residue (35 mg.) consisting of stout rods was recrystallized from $CHCl_3$, then from light petroleum. The product was obtained as thick needles of m.p. 202°. It did not depress the m.p. of authentic cinnamic acid dibromide on admixture. The presence of cinnamic acid as a second metabolite is thus confirmed.

Treatment of the extracted ether solution; isolation of 5-methoxycoumarone

Removal of the solvent from the extracted ether solution left a golden-brown syrup (2·1 g.) which rapidly crystallized on cooling. On dissolution in methanol and dilution with

Table 1. *Cultural details and yields of volatile products of* Stereum subpileatum, *strain F.P.R.L. no.* 271A

No. of flasks	Medium	Period of incubation at 24° (weeks)	Final pH	Residual glucose (%)	Wt. of ether extract of steam-distillate (g.)	Wt. (g.) and m.p. of crude 5-methoxy-coumarone	Wt. of crude cinnamic acid (g.)	Wt. of crude cinnamic aldehyde (g.)	Wt. of non-crystalline residue (g.)
18	Malt	11	4·2	—	1·2	0·56 (28–30°)	0·10	0·12	0·30
21	Synthetic	10	4·6	0·30	1·0	0·23 (29–31°)	0·17	0·33	0·30
50	Malt	10	4·4	—	3·1	1·4 (26–28°)	0·46	0·50	0·60

water, a light-brown oil separated which rapidly hardened on seeding. It was collected, washed with aqueous methanol and dried (1·2 g., m.p. 26–28°). Dilution of the mother liquor gave an oily solid from which further crystals were obtained by pressing on a porous plate. No further solid could be obtained by extraction of the porous plate or from the mother liquors. Recrystallization of the solid material from aqueous methanol afforded colourless shining leaflets of 5-methoxycoumarone of constant m.p. (34°); light-absorption max.: 221, 247, 293, 300·5 mμ in ethanol (log ε, 3·76, 3·99, 3·59, 3·55 respectively [Found: C, 72·9, 73·0; H, 5·6, 5·5; O, 21·4; OMe, 21·0. $C_9H_8O_2$ requires C, 72·95; H, 5·4; O, 21·6; OMe, 20·9%]. 5-Methoxycoumarone is only slightly soluble in cold but more soluble in hot water. It is only slightly soluble in aqueous NaOH. With cold conc. H_2SO_4 it gives a yellowish brown colour, with rapid charring. It decolorizes bromine in CS_2 and $KMnO_4$ in acetone. It has the characteristic odour produced by the cultures. The m.p. of authentic 5-methoxycoumarone (34°) synthesized for comparison was not depressed on admixture with this material. The ultraviolet-absorption spectra of the natural and synthetic products are also in close agreement.

Preparation of bromo derivatives of 5-methoxycoumarone

The natural 5-methoxycoumarone (0·3 g.) was dissolved in CS_2 (2 ml.) and cooled in an ice–salt bath so that the temperature did not rise above −5°. With vigorous stirring bromine (0·33 g., equivalent to 2 atoms of Br/mol.) in CS_2 (2 ml.) was added slowly. Fumes of HBr were evolved. The solvent was removed at room temperature and the residue was dried in a vacuum. Needle-shaped crystals formed in the greenish yellow syrupy residue. The material was taken up in light petroleum (10 ml.) which was decanted from a gummy residue. On evaporation of the petroleum, lemon-yellow needles (50 mg., m.p. 63–65°) of the 2:3:x-*tribromo-5-methoxycoumaran* were obtained. By further crystallizations and sublimation in a vacuum the m.p. was raised to 80° (Found: C, 28·3; H, 1·95; OMe, 8·4. $C_9H_7O_2Br_3$ requires C, 27·9; H, 1·8; OMe, 8·0%). This tribromo compound showed no depression in m.p. when mixed with the yellow product similarly prepared from authentic 5-methoxycoumarone.

The residues from the crystallizations were combined (23 mg., m.p. 70–72°). Recrystallization from light petroleum and sublimation in a vacuum afforded 16 mg. of almost colourless needles of x-*bromo-5-methoxycoumarone*, m.p. 76° (Found: C, 47·7; H, 3·2 (Schoeller); Br, 35·8; OMe, 13·75. $C_9H_7O_2Br$ requires C, 47·6; H, 3·1; Br, 35·2; OMe, 13·7%). This product produced no depression in m.p. when mixed with the colourless monobromo derivative of authentic 5-methoxycoumarone. The third metabolic product isolated is therefore 5-methoxycoumarone.

Antibacterial activity of 5-methoxycoumarone

5-Methoxycoumarone (25 mg.) was dissolved by warming in sterile water (25 ml.) and quickly cooled. The solution remained clear long enough for dilutions in sterile heart broth–glucose (2%) from 1:2000 to 1:10 000 to be made. Two sets of broth tubes were inoculated with 24 hr. cultures of *Staphylococcus aureus* N.C.T.C. 6571 and *Escherichia coli* N.C.T.C. 86 respectively and incubated at 37° for 24 hr.

The inhibition of *Staph. aureus* was complete at 1:5000, partial at 1:8000 and nil at 1:10 000. With *Esch. coli* inhibition was complete at 1:5000, nil at 1:8000. Thus 5-methoxycoumarone has only weak antibacterial activity.

Syntheses

5-Methoxycoumarilic acid (*cf. Tanaka*, 1951). 5-Methoxysalicylaldehyde (3 g.) prepared from quinol monomethyl ether by the method of Rubenstein (1925), diethylbromomalonate (5 g.), anhydrous K_2CO_3 (3 g.) and ethyl methyl ketone (10 ml.) were refluxed together on a steam-bath for 5 hr. The main part of the solvent was distilled off, the residue was mixed with water, acidified with 2N-H_2SO_4 and the solution was extracted six times with one-quarter of its volume of ether. The residue from the ether (7·1 g.), a dark-brown syrup, was treated with ethanolic KOH (2 g./20 ml.) and refluxed for 1 hr. The product was dissolved in water and acidified with 2N-H_2SO_4. Amorphous light-brown material separated [0·8 g., m.p. 163–167° (decomp.)] and was collected. Ether extraction of the filtrate gave a syrup (2·3 g.). The hydrolysis with alkali was repeated on this material since it was calculated that the amount of alkali used (after Tanaka) in the first hydrolysis of the intermediate was insufficient. Further amorphous material (1 g.) was obtained, but not examined.

The filtrate from the first hydrolysis, which had been extracted with ether, when boiled and cooled deposited colourless needles. By a combination of boiling, cooling and concentrating under reduced pressure crystalline material (1·28 g.) of m.p. 214–215° was obtained. This was the required 5-methoxycoumarilic acid for which Tanaka gives the m.p. 212–213°. The filtrate from the second hydrolysis, on similar treatment, afforded a further quantity (0·65 g.) of m.p. 212–214°.

5-Methoxycoumarone. 5-Methoxycoumarilic acid (0·5 g.), Cu powder (0·11 g.) and quinoline (10 ml.) were refluxed for 30 min. After cooling, the mixture was diluted with ether, filtered from the Cu and extracted five times with 0·5 vol. of 2N-HCl and twice with water. Removal of the ether left a golden-brown syrup (0·36 g.). By treatment of this syrup dissolved in ethanol with water a slightly sticky solid (0·3 g., m.p. 32–33°) was obtained, which was purified by distillation at 70–75°/16 mm. The product, finally crystallized from ethanol–water (1:1), was in the form of colourless irregular plates of constant m.p. (34°), yield 0·27 g.; light-absorption max., 220, 246, 293, 300·5 mμ in ethanol; log ε, 3·77, 3·98, 3·57, 3·51 respectively.

Bromination of 5-methoxycoumarone. The method used, based on that of Stoermer & Kahlert (1902) for the preparation of coumarone dibromide, has already been described in reference to the natural product. Two bromo-derivatives were again obtained. These were (1) 2:3:x-Tribromo-5-methoxycoumaran, yellow needles m.p. 79–80° [Found: C, 28·8; H, 2·0; Br, 61·5; OMe, 7·9 (Schoeller); C, 27·5; H, 2·3; Br, 61·1. Mol.wt. (cryoscopic in camphor), 376. $C_9H_7O_2Br_3$ requires C, 27·9; H, 1·8; Br, 62·0; OMe, 8·0%; mol.wt. 387]. (2) x-Bromo-5-methoxycoumarone, colourless irregular plates, m.p. 76° [Found: C, 47·3, 48·1; H, 3·4, 3·3; Br, 33·1, 34·4; OMe, 13·6 (Schoeller); C, 47·8; H, 3·2; Br, 36·4. $C_9H_7O_2Br$ requires C, 47·6; H, 3·1; Br, 35·2; OMe, 13·7%].

SUMMARY

1. *Stereum subpileatum* Berk. & Curt., a basidiomycete found growing in Persian-oak beer barrels and causing contamination of the beer, was cultured on a malt medium and on a glucose–Marmite–salts medium. The steam-volatile metabolic products, which were qualitatively the same on both media, were examined.

2. Three metabolic products were isolated and identified: cinnamaldehyde, cinnamic acid and 5-methoxycoumarone. 5-Methoxycoumarone when treated with bromine in carbon disulphide afforded two crystalline products: (*a*) colourless x-bromo-5-methoxycoumarone, m.p. 76° and (*b*) yellow 2:3:x-tribromo-5-methoxycoumaran, m.p. 80°.

3. 5-Methoxycoumarone is the product mainly responsible for the penetrating characteristic odour of the fungus.

4. 5-Methoxycoumarone exhibited only weak antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

We are indebted to Professor H. Rapoport and the University of Berkeley, California, for the C, H and O determinations on 5-methoxycoumarone. The Uvispek spectrophotometer used in this work was purchased by means of a grant from the Central Research Fund of London University.

REFERENCES

Birkinshaw, J. H. (1953). *Annu. Rev. Biochem.* **22**, 379.

Birkinshaw, J. H. & Chaplen, P. (1955). *Biochem. J.* **60**, 255.

Birkinshaw, J. H. & Findlay, W. P. K. (1940). *Biochem. J.* **34**, 82.

Long, W. H. (1915). *J. agric. Res.* **5**, (10), 421.

Posternak, T. (1940). *Helv. chim. acta*, **23**, 1046.

Rubenstein, L. (1925). *J. chem. Soc.* **127**, 1998.

Stoermer, R. (1900). *Liebigs Ann.* **312**, 237.

Stoermer, R. & Kahlert, B. (1902). *Ber. dtsch. chem. Ges.* **35**, 1633.

Tanaka, S. (1951). *J. Amer. chem. Soc.* **73**, 872.